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MEDICAL CENTER

LOYOLA UNIVERSITY CHICAGO

IDENTIFICATION, ENRICHMENT, AND CYTOKINE PRODUCTION OF MURINE BONE MARROW STROMAL CELLS

A DISSERTATION SUBMITTED TO

THE FACULTY OF THE GRADUATE SCHOOL OF

LOYOLA UNIVERSITY CHICAGO

IN PARTIAL FULFILLMENT OF THE REQUIREMENT OF THE DEGREE OF

DOCTOR OF PHILOSOPHY

DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY

BY

PHILLIP E. FUNK

CHICAGO, ILLINOIS

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LIST OF ABBREVIATIONS

AChE	acetyl choline esterase
Acid Phos.	acid phosphatase
Alk. Phos.	alkaline phosphatase
BSA	bovine serum albumin
°C	degrees Celcius
CAFC	cobblestone-area forming cell
CD	cluster of differentiation
CFU	colony-forming unit
Chloro. Estr.	chlororacetate esterase
CLA	common leukocyte antigen
cpm	counts per minute
DAB	diaminobenzidine
EPO	erythropoietin
5-FU	5 fluorouracil
FBS	fetal bovine serum
FACS	fluorescence activated cell sorter
FITC	fluorescein isothiocyanate
G-CSF	granulocyte colony

	stimulating factor
GM-CSF	granulocyte-monocyte colony stimulating factor
^{3}H-Tdr	tritiated thymidine deoxyribose
Ig	immunoglobulin
IGF-1	insulin-like growth factor 1
IL	interleukin
i.v.	intravenous
KL	kit ligand
LTBMC	long-term bone marrow culture
LTBMC-B	long-term bone marrow culture supporting B lymphopoiesis
LTBMC-M	long-term bone marrow culture supporting myelopoiesis
mAb	monoclonal antibody
mM	millimolar
M	molar
ml	milliliter
μl	microliter
M-CSF	monocyte colony stimulating

	factor
Naph. Estr.	naphthyl esterase
OC	osteocalcin
PBS	phosphate buffered saline
rpm	revolutions per minute
S.D.	standard deviation
TdT	terminal deoxynucleotidyl transferase
TRITC	thiomethyl rhodamine isothiocyanate
U	unit
UEA	ulex europaeus agglutinin 1
VCAM1	vascular cell adhesion molecule 1
VLA-4	very late antigen 4

Chapter I

INTRODUCTION AND REVIEW OF RELATED LITERATURE

Rationale and Objectives

The bone marrow is the primary site of blood cell production, or hemopoiesis, in post natal mammals. Blood cells develop within the marrow in association with other, nonhemopoietic cell types; including, endothelial cells, reticular cells, osteoblasts, and bone lining cells. Some of these nonhemopoietic cells are believed to provide the supportive microenvironment for blood cell development. This microenvironment can be at least partially reconstituted in long-term bone marrow cultures, allowing blood cells to be produced *in vitro* for several months. Hemopoietic differentiation in these cultures is dependent on the presence of a large adherent cell called a stromal cell. Stromal cells *in vitro* express adhesion molecules that serve to bind the hemopoietic precursors to the stromal cell surface. Inhibition of direct contact with stromal cells ablates hemopoiesis in culture. As well, stromal cells produce a variety of cytokines that can influence hemopoietic development. These facts argue that stromal cells play a pivotal role in controlling blood cell development. However, *in vitro* derived stromal cell lines are heterogeneous by several criteria, raising questions about the phenotype and function of the stromal cell counterpart in the marrow. Alternative hypotheses of distinct stromal cell subsets performing different functions versus a single cell type with a highly plastic phenotype have been proposed. The best avenue to study the biologic activity of any cell type is to study it *in vivo*. However, there is no discrete marker of stromal cells to identify and study them *in vivo*. The aims of this dissertation are to identify and enrich stromal cells directly from bone marrow cell suspensions using a combination of less specific markers that have been described and examine their role in supporting lymphoid

differentiation through the production of cytokines. These studies are singular in their attempt to study stromal cells without *in vitro* culture and will provide a much needed link between *in vitro* and *in vivo* studies of hemopoiesis. The availability of normal, uncultured stromal cells is needed to understand the ways in which they regulate hemopoiesis and are themselves regulated by the requirements of the organism. Such studies are important to begin analyzing the basis of hemopoiesis and the ways it can go awry, as in leukemia, hemopoietic dysfunction, and autoimmunity.

I. Overview of Hemopoiesis

Blood cells must be continually replaced because they have a finite lifetime in the circulation. Red blood cells gradually lose their integrity, platelets are lost with even minor blood loss, and myeloid cells are destroyed in defending the organism against invading pathogens. It is estimated that the entire pool of red blood cells in the body must be replaced every 120 days and granulocytic cells have a lifespan in the circulation of only 6 to 7 hours (1). **Hemopoiesis** is the process by which these distinct lineages of blood cells are produced from a common precursor. Hemopoiesis starts in 'blood islands' of the embryonic yolk sack, then migrates to the fetal liver, and subsequently to postnatal **bone marrow** (2). While the marrow remains the exclusive site of hemopoiesis normally, in times of stress, such as bone marrow failure, hemopoiesis can move again to the spleen or liver (extramedullary hemopoiesis)(3). In mouse the marrow of the long bones, e.g. femur, remains hemopoietic through adulthood, whereas in humans the volume of hemopoietic marrow generally decreases with age, being confined to the flat bones of the ribs, sternum, vertebrae, and iliac crest (4). Nonhemopoietic marrow

accumulates fat, consequently being called yellow marrow (4). Again, in time of increased hemopoietic demand, this yellow marrow will convert back to red, actively hemopoietic, marrow (3).

Hemopoiesis is a normal, homeostatic process and, as such, must be tightly regulated. Aberrations in hemopoiesis can be quite severe, ranging from anemia caused by a deficit in erythrocyte synthesis to leukemia caused by unrestricted growth of lymphocytic or myelocytic cells or their precursors. In these examples, the results of aberrant regulation show that either decreased or increased production of blood cells can be equally detrimental to the individual. A normal physiologic example of such regulation is found in the production of red blood cells. Erythrocyte formation was found to be regulated by a circulating factor as long ago as 1906 (5), a factor now known as erythropoietin (EPO)(6). Lowered oxygen tension in the blood is sensed by cells in the kidney, prompting them to release EPO into the circulation where it travels to the marrow to stimulate erythroid progenitors (7). Similarly, the inflammatory cytokine IL-1 has been found to affect the release of granulocytes from marrow reserves (8) and could indirectly cause increased proliferation of myeloid progenitors (9-15). In addition to control via the circulation, the marrow is innervated, potentially rendering it sensitive to direct stimulation from the nervous system (16-19). Webber and colleagues showed that electro-stimulation of the lumbar trunk in rats led to increased release of reticulocytes, neutrophils, and lymphocytes from the marrow (20,21).

A. Stem Cells

The common precursor cell from which all hemopoietic lineages arise is called

a **pluripotent hemopoietic stem cell (PHSC).** The PHSC is the pinnacle of the hemopoietic hierarchy. A unique property of the PHSC is its capacity for self renewal, that is, the stem cell compartment remains constant throughout the lifetime despite continuous division of the PHSC to generate new blood cells (22,23). PHSC divide very slowly but their immediate progeny undergo extensive proliferation, increasing the number of cells entering hemopoietic differentiation pathways. These rapidly dividing daughters of the PHSC retain their capacity to differentiate into many types of blood cells. The PHSC daughters and their progeny form a progression of cells with slightly differing proliferative capacity and gradually restricted differentiation options. These cells enter into lineage specific differentiation pathways, eventually forming the morphologically-identifiable stages of hemopoietic development characterized in the marrow (22).

Defining the PHSC has been a long-standing, yet elusive, goal of many researchers. A variety of methods have been used to isolate and functionally define PHSC. Cell surface markers often used to enrich mouse PHSC include Thy1, c-kit, AA4.1, SCA-1 (Ly6A/E), CD4, and binding of wheat germ agglutinin (WGA) (22). However, these markers are not exclusive to PHSC so rather complicated, multiparameter cell separations have been employed. Purification of human PHSC is typically based on their expression of the CD34 antigen (22). Equally important is the assay system used to detect PHSC (23). The first definition of stem cell activity was the capacity to form colonies of hemopoietic cells on the spleen (CFU-S) of lethally irradiated animals (24). However, it has recently become clear that CFU-S represent a

more restricted type of hemopoietic cell, not capable of resulting in long-term repopulation of the recipient's hemopoietic organs, the true measure of a PHSC (23). Recently, a novel way of assessing stem cell activity without the extended time required for reconstitution of an irradiated animal was developed. In this assay cells are placed in contact with a feeder layer of adherent bone marrow cells. Very primitive hemopoietic precursors and putatively even PHSC will proliferate in the cultures, forming foci of cells attached to the adherent cells. The amount of time in culture required for such proliferating cells to appear correlates with their hemopoietic primitiveness (25,26,27).

B. B Lymphocyte Development

B lymphocytes, the cells which produce antibody (immunoglobulin, Ig) molecules, are a lineage of hemopoietic cell produced within the bone marrow from PHSC. Unique antibody molecules are assembled during B cell differentiation by the rearrangement of gene segments encoding the antigen combining site of the antibody molecule (28). The hallmark of B cells is expression of the B220 cell surface antigen (CD45RA) which marks all B lineage committed cells in the bone marrow (29,30). Immediately before expression of B220 on the cell surface, the enzyme terminal deoxynucleotidyl transferase (TdT) is expressed in the nucleus. This enzyme is thought to mediate addition of N-region nucleotides into the junctions of Ig heavy chain (IgH) rearrangements (31). After functional rearrangement of an IgH allele is complete the IgHμ chain is expressed in the cytoplasm of the cell (cμ+) and is detectable by immunofluorescence (32). Interleukin-7 is a central growth factor in B cell development. Early cells immediately after

expression of B220 (CD45RA) require the presence of accessory stromal cells and IL-7 for survival (33). Somewhat more mature cells that have begun to express cμ can proliferate in response to IL-7 alone (34,35). Since there is extensive cell loss at the rearrangement steps, it is thought that IL-7 mediated proliferation serves to expand the number of cells entering the stage of Ig light chain (IgL) rearrangement (31). As well, it has been suggested that expression of the functionally rearranged IgH chain complexed with the surrogate-light chain molecules λ5 and VpreB is necessary to continue differentiation (36). Other cytokines, c-kit ligand (also called SCF, MGF, SlF)(37,38,39, this dissertation) and insulin-like growth factor-1 (IGF-1)(40, this dissertation), can also impact on B lineage differentiation. Upon successful recombination of light chain alleles a functional Ig molecule can be expressed on the cell surface. The B cell can then be called an immature B lymphocyte. With further maturation the cell will express Ig molecules of both μ and δ isotypes, both containing identical antigen combining sites, and exit the marrow for the secondary lymphoid organs (31). Detection of each of these stages of B cell development is possible, making it feasible to dissect the differentiation pathway stage by stage (30,32-35).

II. The Microenvironment

All lineages of blood cells originate in the marrow but there is no obvious functional or structural separation into compartments as in other tissues; cells of all lineages are seen scattered throughout the marrow. The bone marrow has been described as, "free-living cells, not constrained by organ defining structures, such as basement membranes, required where specific anatomical relationships underline function."(41)

Only a gross level of order has been found in bone marrow, Lord *et al.* (42,43) found that CFU-S are found preferentially in peripheral marrow, closer to the bone surface. Similarly, Osmond *et al.* (44) and Hermans *et al.* (45) found the earliest B cell precursors chiefly in peripheral location in the marrow. To explain this indiscriminate structure, Trentin and colleagues proposed the **microenvironmental hypothesis** (46). Their hypothesis holds that developing blood cells respond to local influences rather than responding directly to systemic signals. These local influences act over a very short radius, allowing many types of cells to be produced in close apposition to one another within one organ. It is these local signals, or microenvironments, that determine the fate of particular blood cells.

Evidence supporting the microenvironmental hypothesis is found in some natural mutations of the mouse. One of the most recognized of these is the *Sl* (Steel) mutation. This animal has a severe macrocytic anemia, affecting many lineages of blood cells, as well as defects in coat color and gamete formation (47,48). Although the anemia is clearly caused by defective hemopoiesis, transplantation of stem cells from normal mice does not cure the anemia (48). Additionally, transfer of stem cells from *Sl* mutants into irradiated normal animals results in repopulation of the recipients with donor (Sl) type cells (48,49). These defects are duplicated in *in vitro* bone marrow cultures (50). These experiments demonstrate that the hemopoietic cells themselves behave normally but that something intrinsic to the animal's bone marrow prevents normal hemopoiesis. Transplantation of long-term cultured stromal cell lines derived from normal animals to replace the *Sl* microenvironment, however, does cure the Sl anemia, verifying that it is

the microenvironment that is defective (51). The defect in these animals resides in the gene for c-kit ligand (also called SCF, MGF, SlF), a growth factor now known to affect a wide variety of hemopoietic cells (52-58). C-kit ligand can be presented as an integral membrane protein as well as secreted into the microenvironment (54,59,60).

The marrow microenvironment can be roughly subdivided into three broad constituents; **cells, growth factors, and extracellular matrix** (61). The cellular component principally refers to the non-hemopoietic mesenchymal cells which form a three-dimensional network supporting and, according to current thought, orchestrating development of blood cells (61,62), as well as blood cells which can reenter and reside in the marrow. The exact components of the cellular microenvironment will be discussed in detail below. Growth factors are proteins secreted by cells which can mediate effects on target cells when bound to their cognate receptors. Such effects include proliferation, alteration in gene expression leading to differentiation, or suppression of proliferation and/or differentiation. In addition, many hemopoietic cells express receptors for more than one type of growth factor. The interaction of factors simultaneously can cause a different response than the factors would separately, often exaggerating the effect in a synergistic fashion. Such interaction adds to the complex interplay of hemopoietic development. The extracellular matrix (ECM) is composed of protein, proteoglycan, and glycosaminoglycan molecules secreted by cells within the marrow cavity. Hemopoietic cells are able to adhere to the ECM via specific receptors and, on binding, these receptors can convey inductive signals to the cells as well as information about the cell's position. Also, secreted growth factors can be bound to ECM components, enabling the

growth factor signal to be precisely localized and imbued with information from surrounding ECM and cellular microenvironmental components (61,63,64). The interplay of these elements can be quite complex, with the end result being the careful orchestration of blood cell development and its attunement to physiologic need.

A. Marrow Structure and the Cells of the Microenvironment

Structurally, mature marrow is composed of an outer sheath of bone remodeling and lining cells and an inner core of hemopoietic tissues drained by a lattice of venous sinuses. Hemopoietic cells develop supported by a three dimensional network of processes formed by reticular cells (65). The structural cells of the marrow which do not develop into blood cells are collectively referred to as **stroma**. These include endothelial cells, reticular cells, bone lining cells, osteoclasts, and osteoblasts. Blood supply to the marrow enters through a nutrient canal. From this vessel, ascending and descending arteries branch out toward the cortical bone. These vessels subsequently reenter the marrow, collecting into the venous sinuses which drain into the central sinus which exits the marrow again via the nutrient canal (61). Nerve fibers run along the circulation to regulate the smooth muscles lining the arteries (61). A number of efferent nerve fibers contact periarterial adventitial cells via gap junctions. Such complexes, called the neuroreticular complex, appear capable of transducing signals form the nervous system, although their function remains unexplored (19).

Endothelial cells line the sinusoids which permeate the marrow and serve to regulate the exit of newly formed cells from the marrow (8,61). These cells are not connected by tight intercellular junctions and this is thought to be a special characteristic

of marrow endothelial cells. Specific factors are believed to affect the release particular cell types. For instance, the C3e component of complement is thought to cause the release of granulocytes. The inflammatory cytokine IL-1 also appears to promote granulocyte release, and EPO has been implicated in the release of reticulocytes (8). In addition to regulating cells leaving the marrow, endothelial cells *in vitro* are capable of producing hemopoietic cytokines (IL-6, G-, M-,and GM-CSF) and could possibly contribute these to the microenvironment *in vivo* (63). Treatment of endothelial cells *in vitro* with IL-1 or TNF leads to increased transcription of the genes for colony stimulating factors, so production of these hemopoietic cytokines could be tied to the circulating levels of cytokines (9,10,11).

One of the main types of nonhemopoietic stroma cell in the marrow is the reticular cell. Two types of reticular cells have been described in bone marrow, both of which express the enzyme marker alkaline phosphatase (66,67). Adventitial reticular cells line the abluminal side of venous sinusoids and extend reticular processes into the marrow. These cells may be contractile, regulating access of maturing blood cells to the endothelium for subsequent exit from the marrow (66). Fibroblastic reticular cells remain fixed within the marrow and predominate in the endosteal areas. Both cells possess oval or rounded nuclei with prominent nucleoli and extend numerous cytoplasmic processes away from the cell body (66). These processes intimately contact hemopoietic cells forming a "spongework" that physically supports them (65). This intimate contact and the possible contractility of adventitial reticular cells has led some to speculate that these cells can actively trap hemopoietic cells and induce their differentiation (65). Both

types of reticular cells appear to have large amounts of rough endoplasmic reticulum, indicative of active protein synthesis for secretion. Reticular cells are associated with fibers of collagens I and III *in vivo*. Both adventitial and fibroblastic reticular cells cans accumulate lipid under certain experimental conditions (4,65,66). Mature adipocytes in the marrow are commonly seen in a parasinal position, making it likely that adventitial reticular cells are capable of becoming adipocytes (8). Unfortunately, no specific markers of marrow reticular cells are available so it has not been possible to identify reticular cells *in vitro* and determine if they influence hemopoiesis in culture. Nevertheless, observational evidence indicates that adventitial and fibroblastic reticular cells in the marrow maintain intimate contacts with hemopoietic cells and may impact on hemopoietic cell development.

Macrophages, although hemopoietic in origin, reside in the marrow where they are thought to play an important role in blood cell development, particularly in phagocytosing expelled nuclei from reticulocytes (62,65) and B cell precursors that have presumably undergone aberrant Ig gene rearrangements (68). Resident marrow macrophages are easily identifiable by their abundant phagolysosomes (62). Resident marrow macrophages are often dendritic in appearance, extending cytoplasmic processes out to contact developing erythroid cells in a formation known as an erythroblastic island (62). Cultured marrow macrophages have been implicated in regulating the terminal stages of B cell development (69,70), however macrophages *in situ* are seldom seen associated with lymphoid cells (62). Macrophages are well known as producers of a wide variety of cytokines and hemopoietic growth factors, including M-, G-, GM-CSF,

IL-1, 3, 6, 8, TNF, and, according to a controversial study by Vogt, EPO (63,71,72).

The endosteal layer, which separates the marrow from the inner surface of the bone, contains an enigmatic cell type called the bone lining cell (73). Bone lining cells are found adjacent to the bone, particularly in heavily trabeculated bone. These cells are morphologically unremarkable, described as elongated and flat with a spindle-shaped nucleus. Dramatic changes occur in the bone lining cells upon treatment of an animal with IL-1. These cells become highly proliferative and will move into the marrow enveloping putative stem cells, interacting with developing hemopoietic cells, and replacing the adventitial reticular cells covering the endothelial lining of the venous sinusoids (74). Bone lining cells may influence early steps of hemopoietic development as they lie in a region known to be rich in CFU-S and primitive B cell precursors (42-5). In addition, Chan and Metcalf reported that bone lining cells are a potent source of colony stimulating factors (75). It has been suggested that bone lining cells may represent a form of mesenchymal stem cell which can generate the hemopoietic-supportive stroma.

Also in the endosteum are osteoblasts and osteoclasts, the cells responsible for forming and resorbing bone, respectively. Based of staining with a variety of macrophage specific monoclonal antibody reagents (76) and their dependence on M-CSF (77), osteoclasts may be hemopoietic cells similar to, but distinct from, macrophages (78,79). Osteoblasts develop from precursors in the endosteum and, once differentiated, are characterized by strong expression of alkaline phosphatase and the bone matrix proteins osteopontin and osteocalcin (80,81). Osteoblasts produce collagens I and III (82)

and may release IGF-1, a stimulator of hemopoietic cells, into the marrow microenvironment (78). These data indicate that osteoblasts, cells thought to be solely involved in the production of bone, may also impact on microenvironmental interactions in the marrow.

In summary, the marrow contains several cell types which are found in close apposition to developing blood cells and, therefore, could form part of the microenvironment that influences blood cell development. These include endothelial cells, reticular cells, macrophages, bone lining cells, and osteoblasts.

Although bone and bone marrow have evolved separately, there is good reason to suspect a unique relationship between these organs (78). The formation of marrow appears to be inextricable from the generation of bone. Even bone formed at ectopic sites or *in vitro* will be hollowed out and filled with hemopoietic marrow (83-87). The activity of these two organs is similarly entwined. Guilliani reported that hypoxia resulted in an increase in marrow volume and consequent decrease in bone mineral density (88). In the human diseases sickle cell anemia and thalassemia, the marrow is very active but the bone becomes osteopenic, with fractures being a common complication of the disease (89-92). Yellow marrow appears to become hemopoietically active during repair of bone fractures as well (78). When bone formation is slowed, as in space flight or in paraplegic individuals, hemopoiesis is reduced (93-95). Autoradiographic evidence suggests that the mesenchymal cells which produce the reticular marrow stroma may also become osteoblastic cells (83).

The origin of the microenvironmental cells has been the subject of some

controversy. The weight of the evidence suggests that microenvironmental cells arise from precursors outside of the hemopoietic lineage (96-98). In studies on the transplantability of the microenvironment Simmons *et al.* reported, "Without exception, marrow derived stromal cells . . . were found to be of host genotype" (98). Using autoradiography to study marrow histogenesis, Sahebekhtiari and Tavassoli found "complete dissociation of marrow stroma and hemopoietic stem cells" (83). Nonetheless, a few investigators have maintained that the cells of the microenvironment are formed from hemopoietic stem cells. Huang and Terstappen recently isolated CD34+ cells from human cord blood and reported that single cells could form both a microenvironment and hemopoietic cells *in vitro* (99). This may reflect a specialized case for fetal cells or they may have isolated phenotypically similar cells that nevertheless have distinct differentiation potentials (100). Work by Simmons *et al.* indicates that human marrow stromal cell precursors express the CD34 antigen but did not determine if these cells made hemopoietic cells as well (101).

Evidence suggests that the nonhemopoietic cells of the marrow arise from a common mesenchymal stem cell. Several authors have used regenerating marrow or marrow formation in ectopically implanted bone to study the histogenesis of the marrow stroma (83-87). When the marrow cavity is initially formed a group of rapidly proliferating fibroblastic cells appears. Autoradiographic labelling indicates that these cells continue to divide for a time, producing osteoblasts in the endosteum and endothelial and reticular cells present within the marrow parenchyma (83). Unfortunately these fibroblastic precursors are rather unremarkable, being described as star- or spindle-

shaped with delicate chromatin pattern and two or more nucleoli (83). To date there are no reports of specific identification of these cells. However, Grigoriadis *et al.* have isolated a clonal cell line from fetal rat calvaria that, when cultured with differentiation inducers such as ascorbic acid or dexamethasone, can differentiate into cells of muscle, fat, cartilage, and bone (102). This cell line may reflect the presence of such a multipotential mesenchymal precursor in the developing bone marrow, but its capacity to support hemopoietic differentiation has remained untested. As well, Freidenstein has found cells derived from the bone marrow which possess the potential to become osteogenic (103). Further examination of such mesenchymal precursors is warranted to understand the developmental process of bone and bone marrow.

B. *In Vitro* Models of the Hemopoietic Microenvironment

The role of nonhemopoietic cells in forming the microenvironment was suggested by the close association of developing blood cells with thin cytoplasmic processes of nonhemopoietic cells in the marrow as seen by both light and electron microscopy (61,62,65,67,68). This supposition was strengthened dramatically with the development of **long-term bone marrow cultures (LTBMC).** In the late 1970's Dexter and coworkers discovered that it was possible to reproduce the mouse bone marrow microenvironment, at least partially, *in vitro* (104). By using carefully controlled culture conditions, including horse serum, hydrocortisone, and 33°C incubation temperature, it was possible to derive cultures that both maintained primitive stem cells and allowed the continuous differentiation of myeloid lineage hemopoietic cells. With regular medium replacement these long-term bone marrow cultures (LTBMC-M) could continue to

produce myeloid cells for several months *in vitro*. Whitlock and coworkers subsequently devised a culture system which supported long-term B lymphopoiesis (LTBMC-B)(105,106). Each of these conditions has been further modified for use with human marrow samples, with limited success (107,108). In these culture systems, long-term hemopoiesis is dependent on the formation of a complex adherent layer of cells. Under LTBMC-M conditions this adherent layer includes macrophages, adipocytes, endothelial cells, and a cell often referred to as a blanket cell, whereas LTBMC-B adherent layers are composed almost exclusively of macrophages and an ill-defined, highly spread adherent cell (3,63,64,104-106,109-111). Developing hemopoietic cells in both culture systems are seen attached to these large, highly spread adherent cells which have come to be called stromal cells.

III. Stromal Cells

The large adherent cells in these cultures have been the subject of scrutiny because of their apparent intimate involvement in hemopoietic development. Inhibition of the physical contact between hemopoietic cells and stromal cells by either diffusion chambers or antibodies to adhesion molecules prevents development of the hemopoietic cells (112-119). These cells have also been shown to produce a variety of growth factors thought to be important in hemopoiesis (3,12,15,63,64,120-124). Several groups have adopted a reductionist approach to the study of stromal cells, deriving cell lines in hopes of reconstructing *in vitro* the minimal requirements for hemopoiesis (3,64,125-136). However, such studies have revealed surprising heterogeneity among stromal cell lines and with primary LTBMC stromal cells. The term stromal cell has come to refer to any

Table 1.-- An Example of a Stromal Cell Classification Scheme Based on Properties of Cloned Cell Lines[a]

Stromal Cell Subtype	Collagen Types	Fibronectin and Laminin	Alkaline Phosphatase	Stem Cell Support in Culture
Endothelial-Adipose	I, IV, V	++	±	+
Fibroblast	I, III	+	++	-
Endothelial-like	IV, V	+	±	-
Fibroendothelial	I, III, IV, V	+	+	-
Macrophage	-	-	-	-

[a] As proposed by Zipori (137).

nonhemopoietic adherent cell derived from bone marrow; cells with widely differing properties also described as stellate, fibroblastoid, endothelial-like, epithelioid, and fibroendothelioid among others (110). Zipori has proposed a classification scheme for cultured stromal cell lines based on their production of extracellular matrix components, hoping to correlate stromal cell lines with cells found normally within the marrow (Table 1)(137). Although well intended, this scheme has probably only furthered the confusion over the lineage derivation of cultured stromal cells as it is not universally applied. Many groups refer to their stromal cells as fibroblastic based on morphology without examining their extracellular matrix production. Additionally, the morphologic definitions used can vary considerably from laboratory to laboratory. The functional relevance of stromal cell lines to normal cells in the marrow is also questionable. Cells described as fibroblastic are commonly cultured from marrow, although there may be few fibroblasts present in the hemopoietic spaces since most fibroblasts are associated with vascular channels (8). Therefore the cultured marrow fibroblasts commonly reported may be from blood vessel derived cells or rare unassociated fibroblasts and any hemopoietic support function seen *in vitro* could be artifactual or a minor aspect of normal hemopoiesis in the marrow.

A. Primary Culture

Stromal cells in primary LTBMC cultures appear homogeneous in several features, notably their characteristic large size and oval nuclei with many nucleoli, the production of collagens I and IV, laminin, and display of the cell surface antigens MECA 10, VCAM1, CD44, and 8.28 (96,110,111,121,132). Primary cultured stromal cells do

not endocytose the acetylated form of LDL, phagocytize latex beads or Ig-coated red blood cells, or express the common leukocyte antigen (CLA) and macrophage cell surface markers (110). These cells express a smooth muscle specific isoform of actin, although it has been suggested that it is present only when the cells are proliferating (138-140). Simply interpreted, the stromal cells seem to be neither fibroblasts nor endothelial cells, but may be related to pericytes, myofibroblasts, or smooth muscle cells. Many groups believe that they are equivalent to the adventitial reticular cells (64). These, as well as endosteal reticular cells, express alkaline phosphatase *in vivo* (66,67). Such apparent homogeneity might reflect only a single cell type mediating all hemopoietic support functions *in vivo*. This single cell could then alter its phenotype in response to environmental changes, consequently altering the number and type of blood cells produced. The ability to switch stromal cell layers from LTBMC-M to LTBMC-B conditions with ensuing production of B lymphocytes on the pre-formed stromal cell layer would appear to support the contention that there is only a single stromal cell type (141).

B. Stromal Cell Lines

As stated previously, many groups have produced stromal cell lines in attempts to understand stromal cell function. A variety of methods have been used to generate these lines, including both repeated subculture (128-136) and direct transformation (125-127). Such cell lines have been helpful in the discovery of new hemopoietic growth factors, such as IL-7, IL-11, and kit ligand (63), and analysis of adhesion mechanisms used by hemopoietic cells (113-117). However, this method has distinct traps in equating a cell line with normal cells present in the marrow. First, transformation of cells often

leads to regression to a fetal phenotype (126). Second, since we envision stromal cells *in vivo* as responding to external stimuli in the regulation of hemopoiesis it is possible that their phenotype *in vitro* could change considerably in reaction to subtle changes in culture conditions. Third, the cloning process could select for rare cells in the starting population. These problems make it difficult to ascertain if stromal cell lines are representative of any, or even some, of the cells normally present in the marrow.

C. Heterogeneity Among Stromal Cells

1. Cell Surface Phenotype

Although stromal cells in primary culture appear homogeneous, stromal cell lines exhibit a great deal of diversity. For instance, the Thy1 antigen is not expressed by primary cells but does appear on some cell lines (3). Some cell lines have been reported to express the common leukocyte antigen (CLA, T200, CD45) or even the pre-B cell aminopeptidase recognized by mAb BP1 (132,133). Selected cells have the capacity to accumulate lipid *in vitro*, but again this is not a universal trait (129,130,134). Adventitial reticular cells, commonly thought to be the *in vivo* equivalent of stromal cells, express alkaline phosphatase activity (66,67), as do a portion of the stromal cells in LTBMC-B (142). However, alkaline phosphatase, as well as reactivity with other histochemical stains varies among stromal cell lines (129). In contrast to stromal cells in primary culture, which have a seemingly uniform cell surface phenotype, stromal cell lines are highly variable, making it difficult to determine if the stromal cell lines are aberrant from the primary cells or represent the outgrowth of minor components of the primary culture population.

2. Hemopoietic Support

Surprisingly, even the ability to support hemopoietic development varies among stromal cell lines (3,64,132). Some groups have isolated cell lines capable of supporting both myelopoiesis and lymphopoiesis *in vitro*, suggesting that only one stromal cell type need be present *in vivo* (131,135). That cell could then modulate its activity in response to environmental cues (141). This view is also supported by the fact that LTBMC-M cultures switched to LTBMC-B conditions will convert to long-term production of B lineage cells. The converse is not true because LTBMC-B cultures do not maintain the primitive precursors needed to initiate myelopoiesis in culture. Other cell lines, however, are more selective in hemopoietic support (127,128). Dorshkind and coworkers have isolated cell lines which support selected stages of B cell development; S17 supports to generation of pre-B cells from early progenitors in the marrow while S10 supports the differentiation of sIg+ B cells from pre-B cells (127,143). The existence of such cell lines has led to speculation that such functionally distinct subtypes are present *in vivo* alongside other conclusions that a single stromal cell type can support all lineages and stages of hemopoiesis. Again, heterogeneity among stromal cell lines has led to apparently conflicting experimental results, making conclusions about the nature of stromal cell support *in vivo* difficult to draw.

3. Stromal Dependent Stages of Hemopoiesis

Evidence exists that only particular stages of hemopoietic development require stromal cell contact. Hardy and coworkers used multiparameter cell sorting to dissect early stages of B cell development and found that very early cells required both contact

with stromal cells and IL-7 whereas later cell type needed only IL-7 to proliferate in culture (33). Similarly, Kierney and Dorshkind showed that lymphoid progenitors capable of initiating lymphopoiesis in culture required physical interaction with stromal cells (112) while Lee *et al.* found that cells produced in culture can respond to IL-7 alone in semi-solid medium (34). The jumbled scattering of cells in bone marrow has made it difficult to examine such preferential associations *in vivo*. Crocker and Gordon, in attempts to study resident bone marrow macrophages, isolated multicellular clusters from murine marrow suspensions (144). Such clusters, in addition to being enriched in macrophages and fibroblastic cells, are enriched in colony-forming cells responsive to GM-CSF. This indicates, at a rough level, an association between early myeloid progenitors and resident macrophages and fibroblasts. Working with human marrow aspirates, Blaszek and colleagues described a structure containing a central adipocyte which serves to organize developing hemopoietic cells which are tightly adherent to it. They termed this unit a Hematon, suggesting that it was centrally involved in hemopoietic cell development (145).

4. Adhesion Molecules on Stromal Cells

Adhesion between hemopoietic cells and stromal cells *in vitro* has recently been the subject of intense study (112-117,146). Studies by Miyake *et al.* have resolved two molecules expressed by stromal cells that are involved in sequestering B cell precursors (113,114,116,117). The first is Pgp-1 (CD44) which is capable of adhesion to the extracellular matrix component hyaluronate (114,117). The addition of antibodies to Pgp-1 to LTBMC will inhibit hemopoiesis in culture if present at the initiation of culture.

Pgp-1 is present on >90% of murine bone marrow cells. Another stromal cell molecule identified by Miyake *et al.* was Vascular Cell Adhesion Molecule 1 (VCAM1)(113,116). Jacobsen *et al.* have reported the presence of VCAM1 on reticular cells within bone marrow as well as some marrow endothelial cells (147). A counter-receptor for VCAM1 is VLA-4, which is expressed on >90% of bone marrow cells. Antibodies to either VCAM1 or VLA-4 will inhibit *in vitro* hemopoiesis even if added after the cultures are actively producing blood cells. This strongly implicates the interaction of VCAM1/VLA-4 in mediating the contact between stromal cells and hemopoietic cells.

VCAM1 was initially characterized and cloned from endothelial cells. VCAM1 is a member of the immunoglobulin-like family of adhesion receptors, existing in either of two forms containing either 6 or 7 Ig-like domains (148,149). VCAM1 is not constitutively expressed on endothelial cells, rather it is upregulated 2 to 4 hours after exposure of the endothelium to inflammatory cytokines such as IL-1 or TNF. When expressed on inflamed endothelium, VCAM1 serves to hold circulating leukocytes, allowing them to eventually migrate into tissue to fight infection. VCAM1 has also been reported to be expressed on dendritic cells in the lymph node and skin and on synovial cells in inflamed joints (150). VCAM1 is a well known adhesion molecule that is expressed on cultured stromal cells and has been strongly implicated in their hemopoietic support function. Its expression on stromal cells in the marrow could represent a marker useful for analyzing fresh stromal cells without the complications that have surrounded work on stromal cell lines.

5. Cytokine Production by Stromal Cells

Stromal cells *in vitro* are known to produce several growth factors which affect hemopoietic development (3,12,15,38,39,40,63,64,120,121,122,143,151). This is one of the most important lines of evidence suggesting that stromal cells influence hemopoietic cell development. Not only do stromal cells constitutively express several growth factors, they are able to modulate growth factor production in response to external stimuli; such as changes in culture conditions (141,152), addition of lithium (153), or the presence of IL-1 (12,15). Again patterns of specific growth factor production vary among cell lines (3). However, transcription of several cytokine genes does not appear to change in LTBMC-B cultures switched to M conditions (154). This would suggest that cytokine production is not the primary means by which stromal cells might regulate production of different hemopoietic cell lineages. IL-3 stimulates early hemopoietic cells and is thought to be involved in stem cell maintenance. However, only one group has been able to detect IL-3 transcripts in stromal cells (63). Other groups have produced cell lines which maintain stem cells *in vitro* with no evidence of IL-3 production (155). Evidence such as this has led Dexter to suggest that, although growth factors influence hemopoietic development, they are not involved in stromal cell-mediated hemopoiesis (123). This directly questions a long-held assumption of hemopoiesis researchers.

Very little has been done to address the production of hemopoietic growth factors in normal marrow, even though the continual production of blood cells occurring there would suggest the presence of such growth factors. Although methods such as *in situ* hybridization and immunocytochemistry would be useful in addressing which cytokines

are produced in the marrow and by what cells, this approach is technically difficult due to the necessity of sectioning through bone and the requirement for electron microscopy to see thin reticular cell processes (156,157). Recently, an ELIspot assay was used to count the number of IL-6 secreting cells in the marrow (158). However, the exact cells producing the IL-6 were not identified in that study, rather, they were negatively selected by FACS. Cytokine production within the marrow is, therefore, a largely unexplored avenue of research.

Three cytokines appear to be produced solely by stromal cells in primary LTBMC-B: IL-7, kit ligand, and M-CSF (121). Two of these, IL-7 and kit ligand, mediate effects directly on B cell precursors (34,35,38,39). IL-7 is a cytokine with a central role in development of both B and T lymphocytes. IL-7 was initially characterized and cloned from a transformed stromal cell line and appears to be very important to the production of lymphocytes in culture (3,34,35,39,64,159). Administration of IL-7 to lymphopenic mice results in a more rapid recovery of lymphocytes, supporting a role for this cytokine in lymphopoiesis *in vivo* (160). Additional evidence for this view is that treatment of animals with antibody that neutralizes IL-7 results in loss of lymphocytes from the marrow (161). This loss occurs at the stage of lymphoid development *in vivo* equivalent with the cells which respond to IL-7 *in vitro*. This evidence suggests that IL-7 is important for lymphoid development in the marrow but the specific cells producing IL-7 in the marrow have not been identified.

Kit ligand (also called Stem Cell Factor [SCF], Mast Cell Growth Factor [MGF],

or Steel Factor [SlF]) is a recently identified cytokine with broad effects, influencing hemopoiesis, melanogenesis, and gametogenesis (47-58). Kit ligand affects many hemopoietic cell types, often acting in synergy with other growth factors (38,39,52). In particular, kit ligand appears to amplify the proliferation of B cell precursors responding to IL-7 (38,39). Administration of an antibody that inhibits the action of kit ligand leads to a dramatic decrease in most hemopoietic lineages in the marrow (162). However, there is a pronounced increase in lymphoid cells, even though kit ligand does appear to affect their development. Kit ligand is produced by cultured stromal cell lines as well as cells in primary culture (38,39,121). Again, despite strong suggestive evidence for kit ligand production in the marrow, no one has identified the cells producing it *in vivo*.

M-CSF production is common among cultured stromal cell lines; in fact, M-CSF is produced constitutively by several different adherent cell types (3,64,121). Based on the presence of M-CSF in stromal cell lines, it has been suggested that M-CSF is important in *in vivo* hemopoiesis, possibly to maintain resident macrophages in the marrow (122). The osteopetrotic mutant mouse is unable to produce M-CSF, leading to a variety of deficits in the monocyte/macrophage lineage of cells (163). However, the anemia of the animals is attributed to decreased marrow volume rather than defective blood cell formation, again with the exception of monocytes/macrophages (164). The expression of M-CSF in a variety of adherent cell types raises the possibility that its expression is an artifact of tissue culture conditions. For this reason, it would be useful to determine if M-CSF is expressed by uncultured stromal cells to gain a better understanding of stromal cell activity *in vivo*.

IV. Summary

In summary, the exact lineage derivation, location, function, and extent of heterogeneity of the stromal cell's *in vivo* counterpart remain largely unknown. Stromal cell lines are quite heterogeneous in cell surface phenotype, growth factor production, adhesion mechanisms used, and capacity to support hemopoietic development. Therefore, it is unlikely that any individual stromal cell line accurately reflects the activity of *in vivo* stromal cells. The phenotypic heterogeneity of stromal cell lines makes it difficult to assign them to any of the non-hemopoietic cell types known to be present in the marrow. Such heterogeneity might reflect heterogeneity in the initial population or phenotypic changes in a highly plastic cell type due to culture adaptation of the transformation process itself. It is also possible that stromal cells in primary cultures represent only a subset of those present *in vivo*. Stromal cell lines have been found to make many growth factors, however, the factors that are truly important in the normal regulation of hematopoiesis are unknown.

To date, stromal cells have principally been used as *in vitro* tools in the study of blood cell development. Very little certain knowledge about the nature of stromal cells themselves and their responses to physiologic stimuli has been forthcoming. The dynamic nature of hemopoiesis and the strong evidence implicating stromal cells in its regulation would seem to make it imperative to understand stromal cells in order to grasp the whole hierarchy of blood cell regulation. As yet there is no way to isolate stromal cells for study directly from the marrow, isolation protocols requiring intervening culture, again leading to the issue of culture adaptation. As well, *in vitro* studies suggest

that particular stages of hemopoiesis require stromal cell contact; whereas, others can develop in the presence of growth factors alone. Whether such contact between particular developmental steps and stromal cells occurs *in vivo* remains unaddressed.

V. Objectives

To paraphrase Tavassoli, 'Hematopoiesis occurs in the bone marrow and not in plastic dishes' (165). In order to gain insight into the normal function of stromal cells it will be necessary to study normal stromal cells. No matter how useful stromal cell lines are in studying certain aspects of hematopoiesis they have not informed us of the lineage derivation, normal growth factor production, and location of stromal cells *in vivo*. **Isolation of fresh stromal cells and analysis of their growth factor production, therefore, are important ways to gain an understanding of hematopoietic regulation. This dissertation aims to examine stromal cells; first, by enriching them directly from bone marrow cell suspensions without intervening culture and second, following isolation, their expression of two lymphopoietic cytokines, IL-7 and kit ligand, will be examined.** Successful completion of this research would allow definition and study of stromal cell phenotype and function without the complication of *in vitro* culture. This will tell us if IL-7 and kit ligand are actually produced by these cells in the marrow. Since M-CSF is produced by all stromal cell lines so far examined I feel that it will be useful to test for production of this growth factor by immunocytochemistry as well.

Experiments such as this are an important first step in the study of stromal cells *in situ*. Study of fresh stromal cells is important in order to comprehend all aspects of

normal hematopoietic regulation. While *in vitro* techniques and cell lines have been very valuable in the study of blood cell production they can only form a part of the entire picture. The results presented here describe attempts to enrich stromal cells directly from fresh marrow, develop ways to identify stromal cells when freshly isolated, and analyze their production of selected cytokines. These are all novel additions to the field of lymphopoiesis and open new avenues of investigation to other researchers. Once normal stromal cells are available it may be possible to develop new and more specific ways to identify them, particularly with monoclonal antibody reagents. The study of defects in the hematopoietic microenvironment will be aided by having access to the aberrant cell types. The mechanisms by which stromal cells sense and respond to their environment and their ensuing effects on hematopoiesis can begin to be studied. These and other areas of research will benefit from this project.

Chapter II

ENRICHMENT OF PRIMARY LYMPHOCYTE-SUPPORTING STROMAL CELLS AND CHARACTERIZATION 0F ASSOCIATED B LYMPHOCYTE PROGENITORS

ABSTRACT

Studies of Whitlock/Witte long term bone marrow cultures have revealed the necessity of two cell types for B-lymphopoiesis, a stem cell and the stromal cell. While a number of stromal cell lines exist they have been found to be heterogeneous with respect to cell surface marker expression and growth factor production. Separation and analysis of fresh bone marrow stromal cells is, therefore, necessary to understand the regulation of lymphopoiesis *in vivo.* Here I report the early stages of such studies, demonstrating that stromal cells, as assessed by morphology and alkaline phosphatase reactivity after short term culture, are enriched in cellular aggregates that can be separated from bone marrow suspensions. Stromal cells are present in aggregates at a frequency of one per thousand cells whereas marrow from which the aggregates have been removed contains only one stromal cell per fifty-thousand cells. These aggregates are able to form Whitlock cultures from greatly reduced numbers of initiating cells, indicating that they contain culturable B lineage precursors as well as stromal cells capable of supporting B-lymphopoiesis. The aggregates appear to be naturally-formed and provide a means to examine native B-cell precursor-stromal cell contacts. Little evidence was found for sequestering of late stage B cell precursors within the aggregates. TdT^+ cells, on the other hand, are approximately three times more frequent in bone marrow aggregates, suggesting close contact between very early B-cell progenitors and stromal cells within the aggregates. The finding that stromal cells are enriched in cellular aggregates is an important first step in the ultimate isolation of these cells from marrow suspensions, which is vital to understanding stromal cell function *in vivo.*

INTRODUCTION

Regulation of B-lymphocyte development has important ramifications for understanding the basic biological events that initiate cell differentiation and how these events are deranged in disease states such as leukemia, hemopoietic dysfunction, and autoimmunity. As in the myeloid lineages, soluble factors have been identified that appear to induce primarily B-progenitor cell proliferation and differentiation in the marrow. These include an early differentiation factor, which can induce expression of the B220 antigen and cytoplasmic μ H chain (143,151), and interleukin 7 (159), which promotes pre B-cell growth. The myeloid CSFs (M-, G-, and GM-), the B-lineage factors, and a lympho-hemopoietic factor [*c-kit* ligand] can be produced *in vitro* by a type of bone marrow adherent cell termed a "stromal cell" (15,38,143,159,166). Specifically, the term "stromal" has been used to describe a morphologically distinct group of very large, highly spread adherent cells present in Whitlock and Dexter long term bone marrow cultures (110,111).

In addition to growth factor production, a population of cultured stromal cells clearly binds early B-lymphocytes in tight clusters on the surface and beneath the flattened stromal membrane (167). Actual contact with stromal cells seems necessary in the early phases of B-cell differentiation (33,112). This cellular adhesion is mediated in part through the VCAM1 molecule, expressed on stromal cells, and the VLA-4 molecule present on the progenitor cell surface (116). Therefore, at least in culture, bone marrow stromal cells appear to provide key regulatory influences necessary for B-cell development.

The primary goal was to isolate stromal cells from fresh bone marrow in order to examine their normal production of hemopoietic growth factors. As an initial approach based in part on the work of Crocker and Gordon (144), it was found that the highest frequency of culturable stromal cells in a mouse bone marrow suspension are found enmeshed in small, cellular aggregates. Isolated aggregates are an enriched source of fresh stromal cells as well as very immature B-lineage cells.

MATERIALS AND METHODS

Animals. 4 to 8 week old female BALB/c mice were purchased from Harlan Laboratories (Indianapolis, IN) and housed at the Animal Research Facility at Loyola University.

Cell Preparation and Long Term Bone Marrow Culture. Bone marrow cells were prepared and cultured according to Whitlock *et al.* (105). Briefly, mice were sacrificed by cervical dislocation and the femurs removed. Marrow was flushed from the femurs with cold RPMI 1640 medium supplemented with 10% fetal bovine serum by inserting a 25 gauge needle attached to a 3 ml syringe. This cell suspension was then centrifuged at 1000rpm for 10 minutes and the cells resuspended by pipeting 10 to 15 times through a 5 or 10 ml glass serological pipet. Cells were resuspended at a density of 1×10^6 cells per ml and plated in 60mm tissue culture dishes, 5ml per dish. Medium used for long term cultures was RPMI 1640 supplemented with 5% selected FBS (Hyclone, Logan UT, Lots 1111985 and 1111638), 2mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, 50 U/ml penicillin, and 50 μg/ml streptomycin.

Isolation of Aggregates. We modified procedures previously reported for isolation of

cell clusters from bone marrow and thymus, principally those of Crocker and Gordon (144) and Wekerle and Ketelson (168), respectively. After centrifugation the bone marrow cells were resuspended at three femur equivalents per ml and vigorously pipetted through a 5 or 10 ml pipet. 1 ml of this suspension was carefully layered over 3 ml of cold FBS in a 5 ml tube. Aggregates were allowed to settle for 15 minutes by placing the tube on ice in a vertical position. After 15 minutes the following fractions were collected with a pasteur pipet: the uppermost 1 ml, which will be referred to as deaggregated marrow; the middle 2 ml, which contained small aggregates and single cells; and the lowermost 1 ml, which contained aggregates estimated to be 20 to 500 cells per aggregate.

Collagenase Digestion. Collagenase grade CLS 3 lot # FOD 671 (Worthington, Freehold, NJ) or Collagenase A (Boehringer Mannheim, Indianapolis, IN) was used to digest aggregates into single cells. Collagenase was used because it is rarely a pure preparation and contains low levels of other lytic enzymes that are useful for digesting the complex matrix of tissues. These activities can have adverse effects on cell surface antigens, however, and it was necessary to screen several lots before an appropriate batch was found. Collagenase FOD 671 was selected from 11 lots of collagenase by screening for effectiveness of aggregate dispersion and for low tryptic activity by monitoring effects on staining with mAb 14.8 by FACS. The neutral protease dispase was also tested but proved to be particularly damaging to cell surface antigens; as little as 0.5U/ml was found to completely remove the B220 antigen from our cell populations. Digestion conditions used were 1 x 10^7 cells per ml in 0.2% collagenase in RPMI 1640 + 10%

FBS for 90 minutes at 37°C and 5% CO_2. Cells were passed through a 25 gauge needle three times during this digestion in order to break up cell clumps. After digestion, cells were washed in cold RPMI + 10% FCS two times before subsequent manipulations.

The percentage of cells in aggregates was calculated as the number of cells in aggregates divided by the number of marrow cells before aggregate isolation and collagenase treatment. This estimate is likely an underestimation of the proportion of cells present in aggregates since we see cell loss during collagenase treatment. I have summarized some initial experiments in working out collagenase digestion conditions to explain how I arrived at this calculation. I have given the cell number per femur before and after collagenase treatment, the number of cells in aggregates after collagenase treament, and an estimate of the fraction of marrow cells in aggregates based on both unfractioned marrow counts (Table 2). In every case the use of collagenase-treated marrow in this calculation increases the estimate, sometimes by nearly 2-fold. Although it is likely that cells are also lost from the aggregates it is impossible to estimate this loss. If one calculated the percent of marrow in aggregates using collagenase treated marrow one must assume that there is equivalent cell loss from each group. One is then also using an estimate of the cellularity of marrow that is decreased. I feel that the most accurate way to estimate the percent of cells in marrow is to use the most accurate count of total marrow cells, i.e. untreated marrow, and accept that it is probably an underestimate because of cell loss from the aggregates. Since cell loss can vary from day to day, this allows a more stable comparison of aggregates from experiment to experiment, and to the use of other batches of collagenase as it becomes necessary.

Table 2.--Estimating the Fraction of Marrow Cells in Aggregates

Experiment	Untreated[a]	Collagenase Treated[a]	Aggregates[a]	Aggregates/ Untreated[b,c]	Aggregates/ Collagenase Treated[d]
1.	9.9	7.6	0.66	0.048	0.087
2.	10.8	8.6	0.58	0.053	0.067
3.	10.1	6.0	0.26	0.026	0.043
4.	20.0	10.9	0.99	0.047	0.086
5.	12.7	12.4	0.58	0.045	0.046
6.	10.4	5.9	0.42	0.040	0.075
7.	8.9	6.9	0.95	0.106	0.138
8.	9.9	6.4	0.10	0.099	0.154
9.	7.8	6.0	0.29	0.037	0.049

[a] X 10^6 per femur. [b] Calculated as the number of cells in aggregates divided by the number of cells in treated marrow. [c] Average value 0.056, within normal range of 0.081 ± 0.025. [d] Calculated as the number of cells in aggregates divided by the number of cells in unfractioned, collagenase treated marrow.

Determination of Stromal Cell Frequency. Cells were counted after collagenase treatment, plated into 60mm tissue culture dishes, and allowed to adhere for 48 hours. 48 hours was previously determined to be sufficient for stromal cells to attach and spread, and thus display their characteristic morphology in culture. Medium used was identical to LTBMC-B medium except were icreased serum concentrations are noted. In some experiments plates were precoated with 20μg/ml fibronectin (Biomedical Technologies, Stoughton, MA), 20μg/ml laminin (Biomedical Technologies), or 20μg/ml of Pronectin F(Protein Polymer Technologies, San Diego, CA). Also, macrophages and monocytes from the marrow begin to actively proliferate in culture at 48 hours, so this time point prevents excess macrophage proliferation from complicating stromal cell scoring (personal observations). After 48 hours the medium was removed and plates were washed 3 times with cold PBS. Cells were then fixed in methanol and stained with Jenner/Geimsa or fixed in citrate buffered acetone and stained for alkaline phosphatase activity. The entire area of each plate was then examined using a phase contrast microscope. Stromal cell frequency was estimated as the ratio of stromal cells per plate to the total number of cells originally plated. Statistical analysis was by t-test.

Flow Cytometry. After collagenase digestion, 1 x 10^6 cells of each group were stained with the primary antibody for 20 minutes in 50 ul of Hanks Balanced Salt Solution with 0.1% BSA. Primary antibodies were M1/9 (anti-T200, Boehringer Mannheim); 14.8 (anti-B220, ATCC); BP1 (gift of Dr. Max Cooper, Birmingham, AL). Cells were then washed and stained with the appropriate secondary F(ab)'2 antibody for 20 minutes: for BP1 this was FITC-labelled goat-anti-mouse IgG (Fc specific) (Jackson Labs, West Grove, PA) and for all others was FITC-labelled mouse-anti-rat Ig (heavy and light

chains) (Jackson Labs). The cells were then washed and 10,000 viable cells were analyzed by a Becton Dickinson FACStar plus. Results are shown with $\log_{10}$ fluorescence intensity on the horizontal axis and relative cell number on the vertical.

Immunoperoxidase Staining. Cytocentrifuge preparations were fixed for 15 minutes in ice cold 95% ethanol with 5% acetic acid. Slides were then washed 3 times in PBS. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol and the slides were again washed 3 times in PBS. At this point the slides were incubated with 10% FCS and then stained with M/K2 for 60 minutes. MK/2 (anti-VCAM1) was a gift of Dr. Paul Kincade, Oklahoma City, OK. Slides were washed 3 times in PBS and stained with biotinylated rabbit-anti-rat Ig (Vector, Burlingame, CA). After 3 more PBS washes slides were stained with the Vector ABC Elite kit (Vector). Horseradish peroxidase enzyme activity was detected by immersing the slides in 0.67mg/ml diaminobenzidene for 15 minutes. Slides were counter stained in hematoxylin, washed, dried, and observed by bright field microscopy.

Immunofluorescence Microscopy. B/pre-B differential immunofluorescence (32) was determined by staining cells in suspension with FITC conjugated goat-anti-mouse μ (Jackson Labs). 10^5 cells were cytocentrifuged onto slides, fixed in ice cold ethanol/acetic acid, and stained with TRITC conjugated goat-anti-mouse μ (Jackson Labs). Slides were then scored for $c\mu^+$ pre-B cells (red fluorescence) and $s\mu^+$ B cells (green and red fluorescence) using a fluorescence photomicroscope. At least 250 cells were scored per slide. Results are presented as means of three slides $\pm$ SD. Detection of terminal deoxynucleotidyl transferase (TdT) in the nucleus was done by using kit no. 1480 (Supertechs, Bethesda, MD). Cytocentrifuge spots were prepared with 10^5 cells.

The cells were fixed with methanol and stained with rabbit-anti-mouse TdT followed by goat-anti-rabbit Ig FITC. At least 600 cells were scored on each slide.

CFU Assays. Following collagenase digestion, 10^5 cells of each group were plated in McCoy's modified 5a medium supplemented as described previously (34). Recombinant IL-7 (Biosource, Westlake Village, CA) was used at 500U/ml (34). Cells were plated in 1 ml volume in 35 x 15 mm Lux Contuor (Nunc, Naperville, IL) dishes. Plates were incubated in 7.5% CO_2 in air at 37°C for 6 days. At that time colonies >20 cells were scored using a dissecting microscope. The data are reported as mean $\pm$ S.D. of at least three plates per group.

^{3}H Thymidine Proliferation Assay. Bone marrow populations were plated at various concentrations in 96 well plates and pulsed with 1 μCi/well of ^{3}H TdR for 18 hours. Cells from each well were then harvested with a PhD cell harvester and counted in a β-scintillation counter. Results are mean $\pm$ SD of three or four wells.

Cell Cycle Analysis. 2-3 x 10^6 cells of each bone marrow population were fixed in 70% ethanol at 4°C overnight. Cells were then incubated briefly with RNase, washed, stained with propidium iodide, and analyzed by a FACStar Plus (166). Data presented are the total percentage of cells in S + G_2M of each population.

RESULTS

Stromal cells are enriched by isolating cell aggregates.

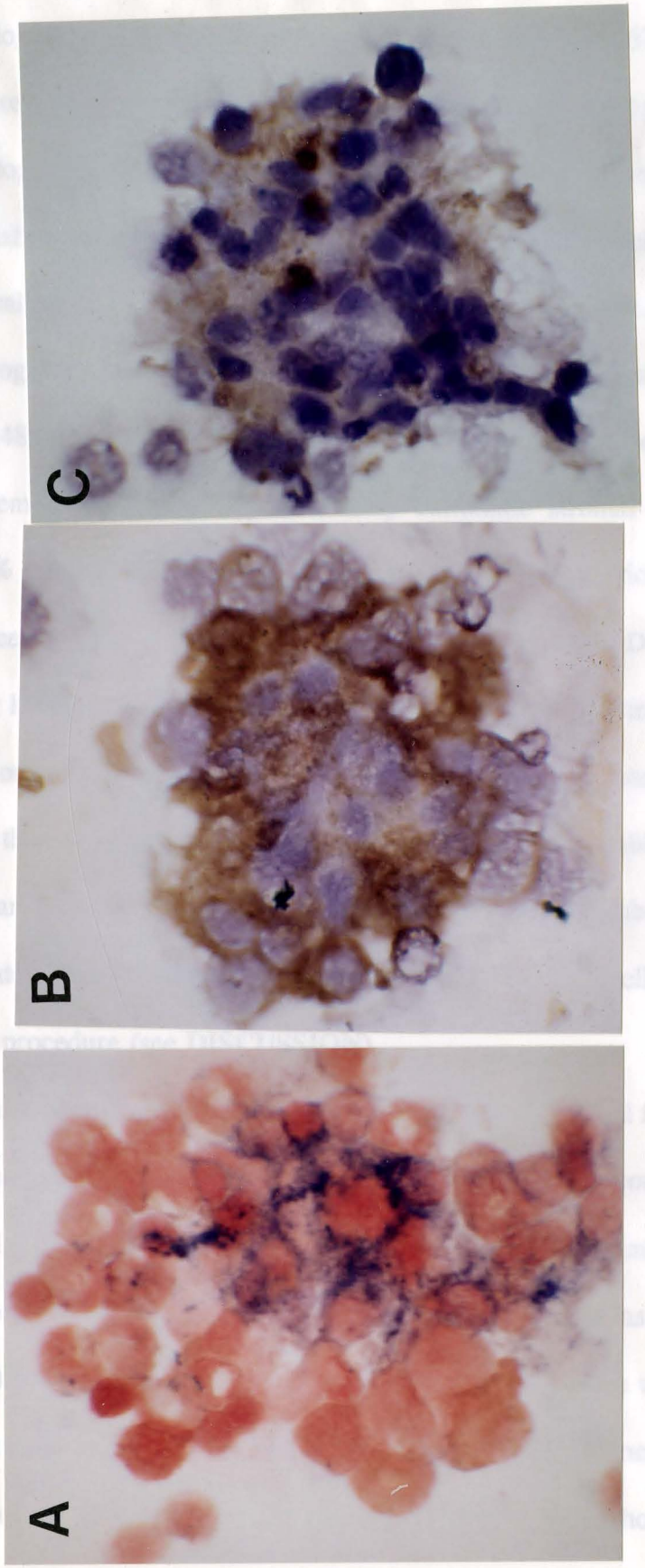
Two days after Whitlock cultures were initiated, most of the adherent cells were observed in discrete foci on the tissue culture dish (Fig. 1A), appearing as if they were growing out from cell clusters or aggregates present in the marrow suspension. This observation led to speculation that most of the stromal cells in an adherent layer of a

Whitlock culture might be obtained merely by separating aggregates from whole marrow suspensions. As a means of isolating cell aggregates, procedures were adapted from Wekerle and Ketelson for enrichment thymic nurse cells (168) and Crocker and Gordon (144) for enrichment bone marrow resident macrophages. Briefly, cellular aggregates were isolated by unit gravity sedimentation over a fetal calf serum layer (see Methods). Collagenase dispersed aggregates contained an average of $8.1 \pm 2.5 \times 10^5$ cells per femur or about 8% of total marrow nucleated cells. This average was derived from 16 independent experiments and is representative of every experiment performed on aggregate isolation.

As evidence that stromal cells were present in aggregates, Figure 1 shows two markers that have been reported on stromal cells *in vivo* and *in vitro*. The first is alkaline phosphatase activity (67,167,169) (Fig. 1A) and the second is the VCAM1 antigen detected by rat mAb M/K2 (113,116) (Fig. 1B). A reticular staining pattern has been reported for both of these proteins *in vivo* (67,113) and this pattern was also seen in the aggregates. Staining was generally confined to aggregates but not every aggregate showed reactivity for alkaline phosphatase or VCAM1. The similarity in staining of marrow sections and aggregates suggests that a native cell architecture from intact marrow may be somewhat preserved during preparation of cell aggregates.

To demonstrate that stromal cells were enriched in the aggregates, short-term (48 hour) culture assay was devised that enabled culturable stromal cells to be counted before significant proliferation of adherent cells occurred. The number of culturable stromal cells present in bone marrow fractions were quantitated based on two previously described characteristics of adherent stromal cells in long-term culture: distinctive

Figure 1. Localization of alkaline phosphatase (A) and VCAM1 (B) in aggregates. A) Aggregate stained for alkaline phosphatase reactivity (blue stain) with safranin background. B) Immunoperoxidase stain for M/K2 (brown areas positive). C) Negative control for M/K2 stain showing background peroxidase activity.


C
B
A

morphology (110,111) and alkaline phosphatase activity (67,167,169) (Table 3). Stromal cells were judged as large, highly spread cells with abundant pseudopodia, a relatively clear cytoplasm, and distinctive oval nuclei with prominent nucleoli. Stromal cells were distinguished from macrophages, the predominant adherent cell type, because the latter were smaller, more fusiform, and had a granular cytoplasm. All cells with stromal morphology retianed their alkaline phosphatase reactivity and VCAM1 expression through 48 hours culture (data not shown). A notable result of these experiments was the extremely low frequency of readily culturable stromal cells in whole marrow (~0.04% of total cells). Aggregates were 2.5 to 3 fold enriched in readily culturable stromal cells, which were present at a frequency of 0.1%. Deaggregated marrow, on the other hand, was substantially depleted of stromal cells. Stromal cells were found to be only one in about fifty-thousand cells in deaggregated marrow, or fifty times less frequent than in aggregates. Therefore most of the recoverable stromal cells found in marrow are present in aggregates. Comparison with the number of stromal cells found in untreated marrow indicated that some of the stromal cells were lost during our isolation procedure (see **DISCUSSION**).

To ensure that our short-term culture assay was optimal for stromal cell detection experiments were performed to test the effects of culture conditions on stromal cell plating. In these experiments collagenase treated unfractioned marrow was used to assess stromal cell plating efficiency. Since the primarily interest was in the stromal cells that support B lymphopoiesis, the conditions of LTBMC-B culture were used. Initially, the serum lot used was altered, reasoning that the serum dependence of LTBMC-B cultures might be due in part to effects on stromal cells (Fig. 2A). Although some variability in

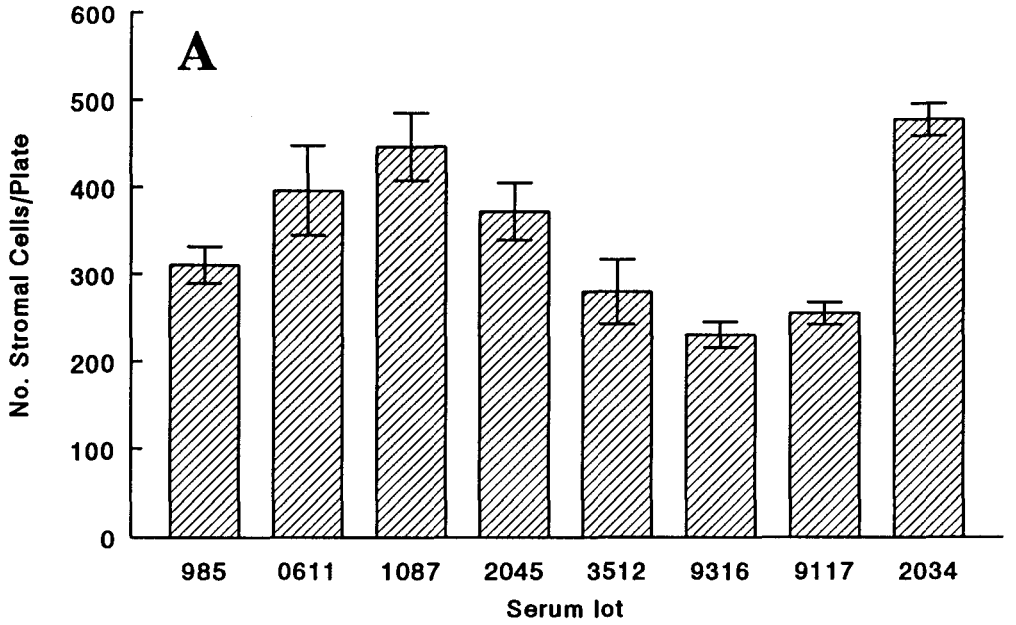
Table 3.--Frequency of Culturable Stromal Cells in Aggregates[a]

	Untreated	Unfractioned[b]	Deaggregated[b]	Aggregates[b]
Experiment 1[c]				
No. of Cells Plated	1×10^6	1×10^6	5×10^6	0.9×10^6
No. of Stromal Cells	331 ± 152	470 ± 177	74 ± 13	1011 ± 177
Frequency of Stromal Cells	3.3×10^{-4}	4.7×10^{-4}	1.5×10^{-5}	1.1×10^{-3}
Experiment 2				
No. of Cells Plated	1×10^6	1×10^6	5×10^6	0.9×10^6
No. of Stromal Cells	407 ± 77	302 ± 16	88 ± 15	976 ± 140
Frequency of Stromal Cells	4.07×10^{-4}	3.02×10^{-4}	1.8×10^{-5}	1.1×10^{-3}

[a] Stromal cells were allowed to adhere in 60mm plates for 48 hours and nonadherent cells were washed off with PBS. Adherent cells were then fixed and stained with Jenner/Giemsa (Expt. 1) or for alkaline phosphatase (Expt. 2) directly on the plate. Stromal cells were then scored by their morphology using a phase contrast microscope. [b] Treated with collagenase. [c] Expt. 1 is representative of two studies.

stromal cell recovery was evident among the serum lots, those that supported lymphopoiesis in culture (lots # 985, 1087, 2045, and 2034) were generally best at stromal cell recovery. There was no statistical difference among these lots. Increasing the concentration of serum in the medium used in the plating assay, using serum lot 2034, did not increase stromal cell plating efficiency (Fig. 2B). The possibility that factors secreted by stromal cells or hemopoietic cells in LTBMC-B might affect stromal cells was also considered. Addition of LTBMC-B conditioned medium did not influence stromal cell plating in the 48 hour culture assay (Fig. 3A). Certain cell types adhere better to cultureware that has been coated with extracellular matrix components. Therefore, fibronectin, laminin, and a synthetic polymer containing multiple repeats of the RGD binding sequence from fibronectin (Pronectin)(Fig. 3B & C) were tested. Fibronectin, although it increased stromal cell recovery occassionally, did not consistently affect stromal cell plating, nor did the conbination of fibronectin with LTBMC-B conditioned medium. Laminin, on the other hand, inhibited stromal cell recovery. Certain cells, notably endothelial cells, require a minimal initial cell density to grow in culture, an effect known as cooperativity (170). Since it has been suggested that stromal cells are endothelial in origin (96) it is possible that cell density might affect stromal cell plating efficiency. The number of stromal cells varies linearly with the number of plated bone marrow cells over a 100-fold range of input cells (Fig. 4), arguing against cooperativity among stromal cells. The previous experiments all used unfractioned marrow as a source of stromal cells. However, it is possible that aggregate stromal cells are a distinctive population, therefore we tested the various conditions on stromal cells derived from collagenase dispersed aggregates (Fig. 5). Again, no

Figure 2. Effects of serum on stromal cell plating. A) Effects of different serum lots. B) Effects of increasing serum concentration. Unfractioned collagenase treated marrow was used as a source of stromal cells, 3.4 X 10^5 cells per plate.


A
No. Stromal Cells/Plate
600
500
400
300
200
100
0
985
0611
1087
2045
3512
9316
9117
2034
Serum lot

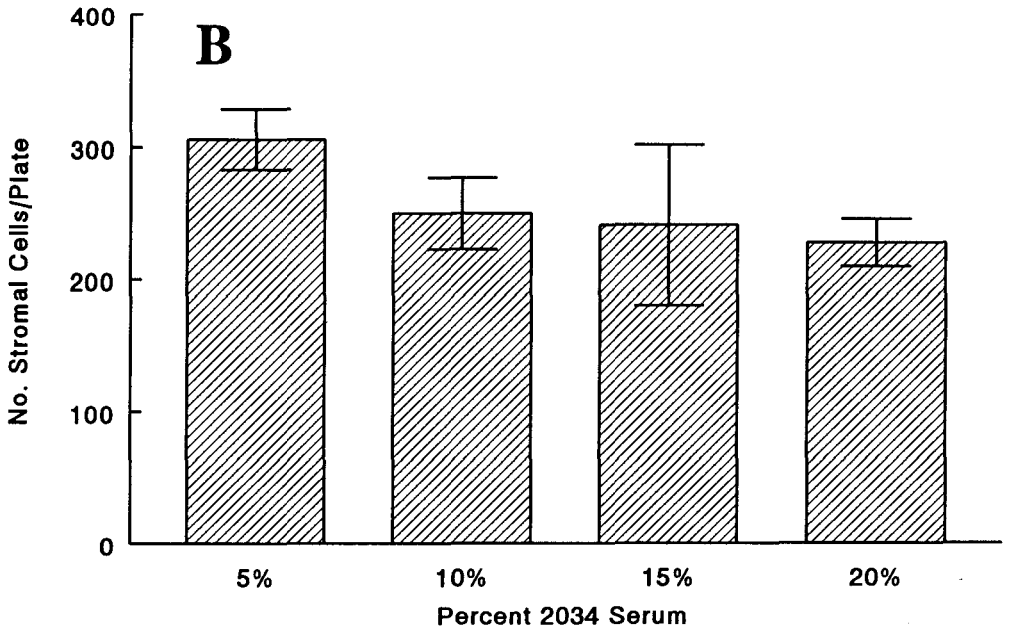
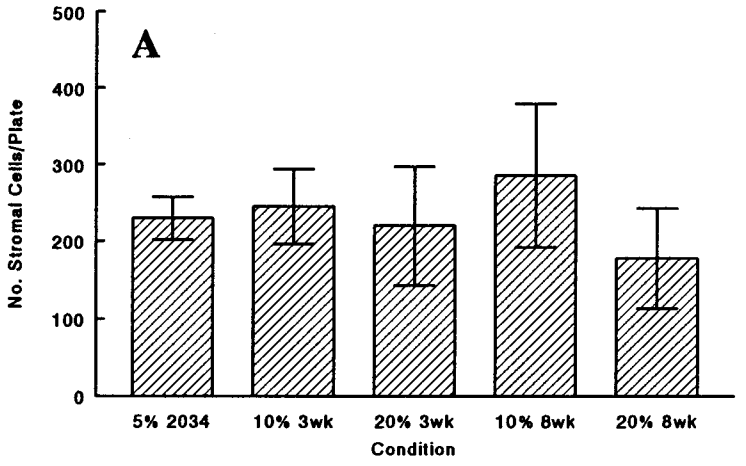
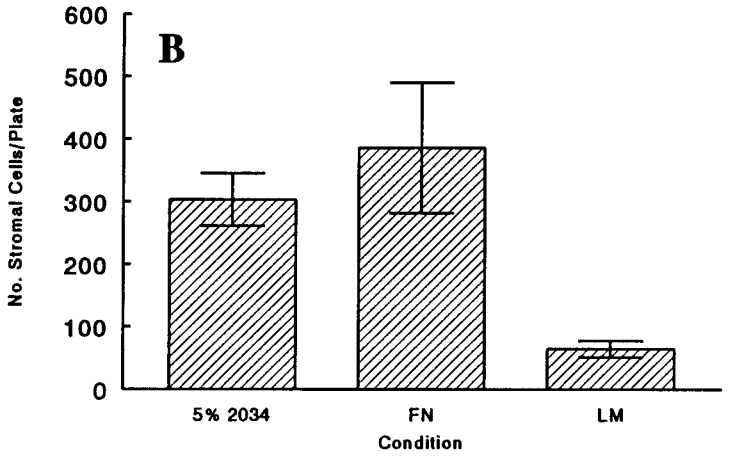

B
No. Stromal Cells/Plate
400
300
200
100
0
5%
10%
15%
20%
Percent 2034 Serum

Figure 3. Effects of medium additions or extracellualr matrix components on stromal cell plating. A) Effects of conditioned media from 3 or 8 week old LTBMC-B cultures. B) Effects of extracellular matrix components fibronectin (FN) and laminin (LM). C) Effects of fibronectin or pronectin (ProN) alone or in combination with 10% conditioned medium from LTBMC-B. Unfractioned collgenase treated marrow was used as a source of stromal cells, 3.4 X 10^5 cells per plate.


A
No. Stromal Cells/Plate
500
400
300
200
100
0
5% 2034
10% 3wk
20% 3wk
10% 8wk
20% 8wk
Condition


B
No. Stromal Cells/Plate
600
500
400
300
200
100
0
5% 2034
FN
LM
Condition

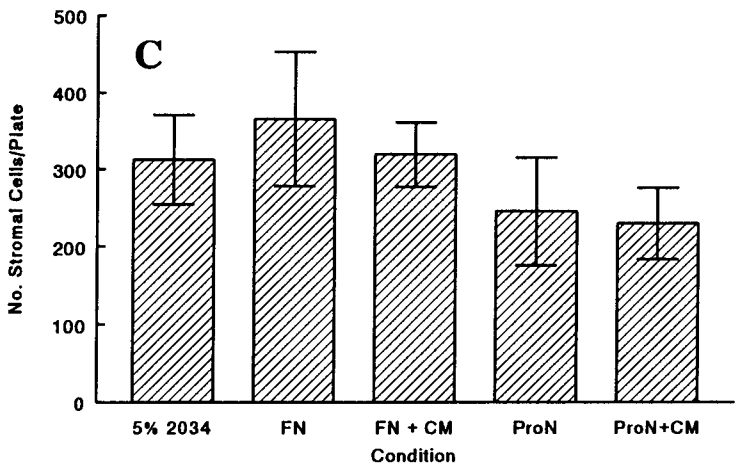

C
No. Stromal Cells/Plate
500
400
300
200
100
0
5% 2034
FN
FN + CM
ProN
ProN+CM
Condition

Figure 4. Effects of cell density on stromal cell plating.

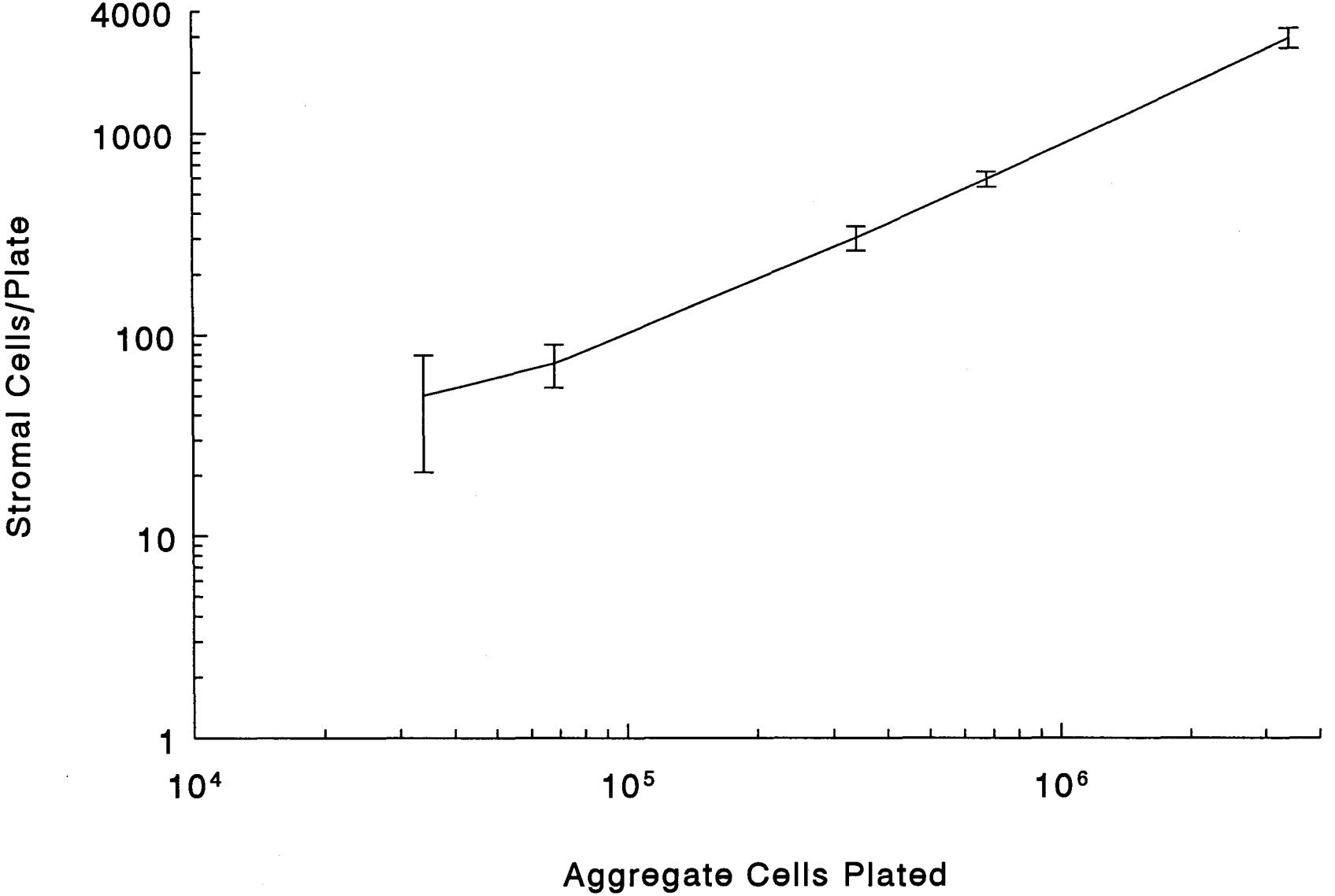
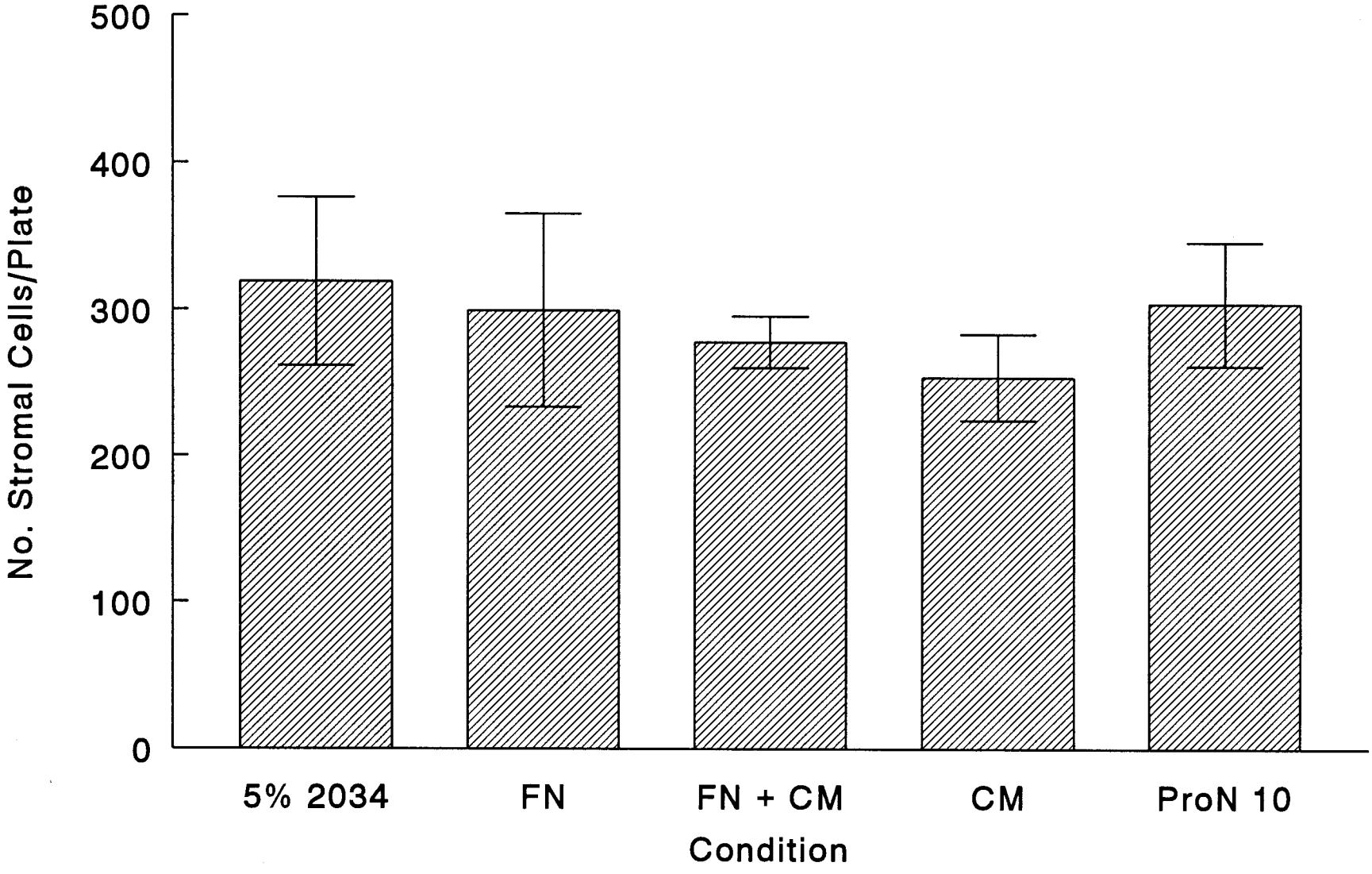

4000
1000
100
10
1
Stromal Cells/Plate
10⁴
10⁵
10⁶
Aggregate Cells Plated

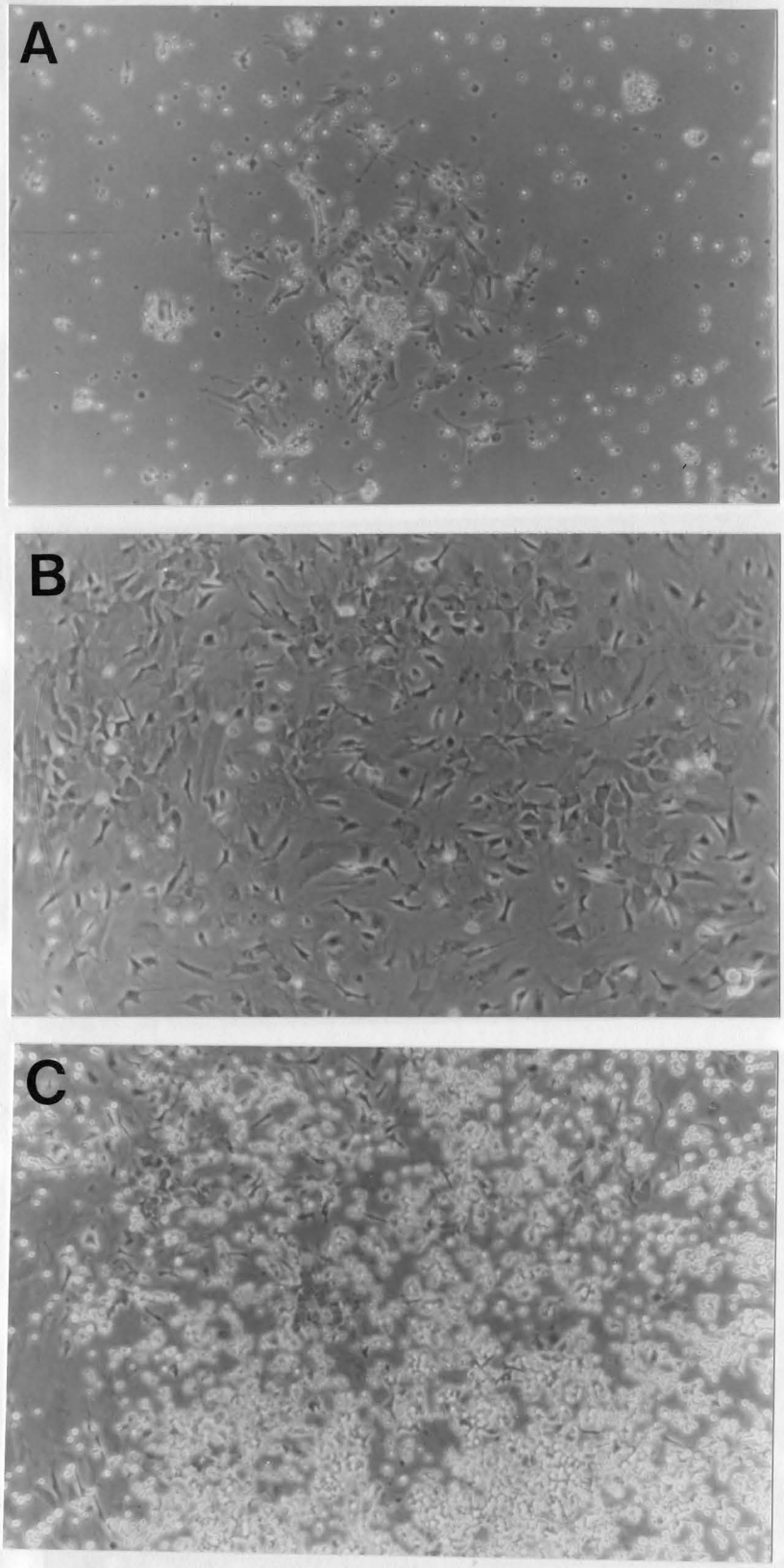
Figure 5. Effects of fibronectin (FN), LTBMC-B conditioned medium (CM), pronectin (ProN), or FN plus CM on plating of stromal cells from collagenase dispersed aggregates, 1.7 X 10^5 cells per plate.

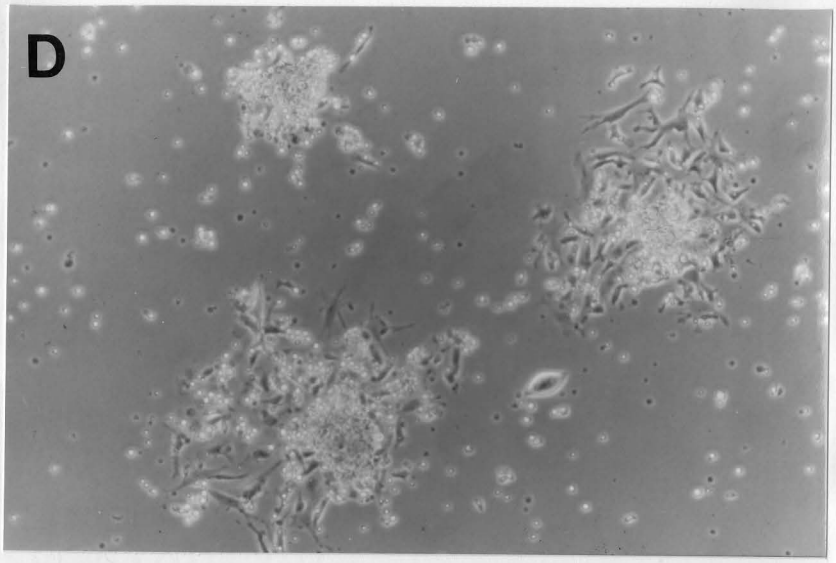

500
400
300
200
100
0
No. Stromal Cells/Plate
5% 2034
FN
FN + CM
CM
ProN 10
Condition

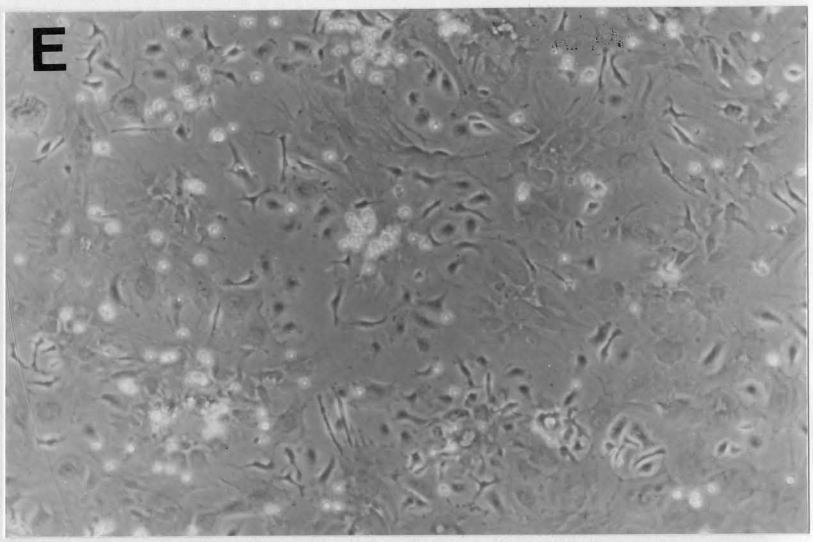
differences were seen among stromal cell plating in each of the tested conditions. Therefore, none of the relatively straightforward tactics commonly used to improve cell plating efficiency in other systems increase the plating of stromal cells. Importantly, these results do not mean that this culture assay detects all stromal cells present, these results to mean stromal cells are not readily aided in plating.

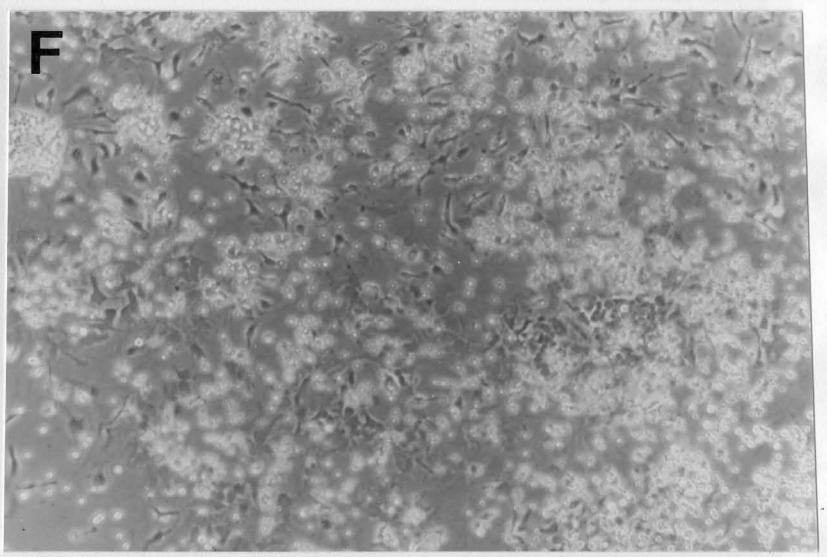
Since the primary interest of the laboratory is lymphopoiesis, it was important to determine if stromal cells derived from aggregates were able to support lymphocyte growth in culture. Whole marrow prepared as in a standard Whitlock culture (105) is plated at a density of $1x10^6$ cells/ml or $5x10^6$ cells/60mm dish. At this concentration cultures typically developed a nearly confluent adherent layer between 14 and 21 days after initiation, and foci of proliferating lymphocytes appeared by day 21 (Fig. 6A-C). Cultures of aggregates followed an identical pattern of culture development, forming a confluent adherent layer around day 17. Lymphocyte foci were visible in aggregate cultures around day 21 (Fig. 6D-F). Notably, collagenase-dispersed aggregates were capable of establishing a Whitlock culture from an initiating cell density of $2\text{-}4x10^5$ cells/ml or $1\text{-}2x10^6$ cells/60mm dish, as few as one-fifth the number of whole marrow cells needed to initiate culture. However, cultures of deaggregated marrow initiated at $5x10^6$ cells/60mm dish proved unable to form Whitlock cultures (Fig. 6G-I). Deaggregated marrow cultures developed a sparse adherent layer and rarely gave rise to lymphocytes even when maintained for up to 3 months. This result would be expected given the data in Table 1 showing the vast depletion of stromal cells in deaggregated marrow. Thus, the bone marrow aggregates contained all of the elements necessary to form a Whitlock culture, and since five-fold fewer cells were capable of forming a

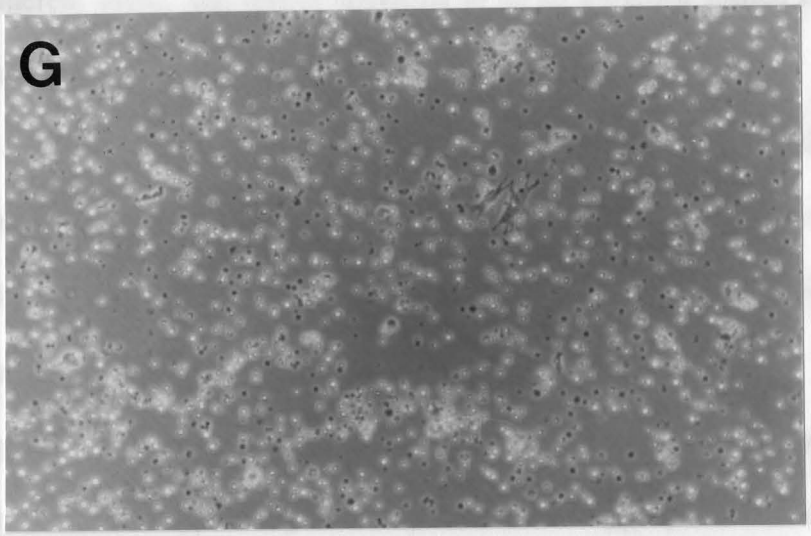
Figure 6. Comparison of Whitlock cultures initiated from marrow fractions. Whitlock culture initiated with whole marrow at: A) 2 days, B) 17 days, and C) 21 days. Cultures initiated with aggregates at: D) 2 days, E) 17 days, and F) 21 days. Cultures initiated with deaggregated marrow at: G) 2 days, H) 17 days, and I) 21 days.

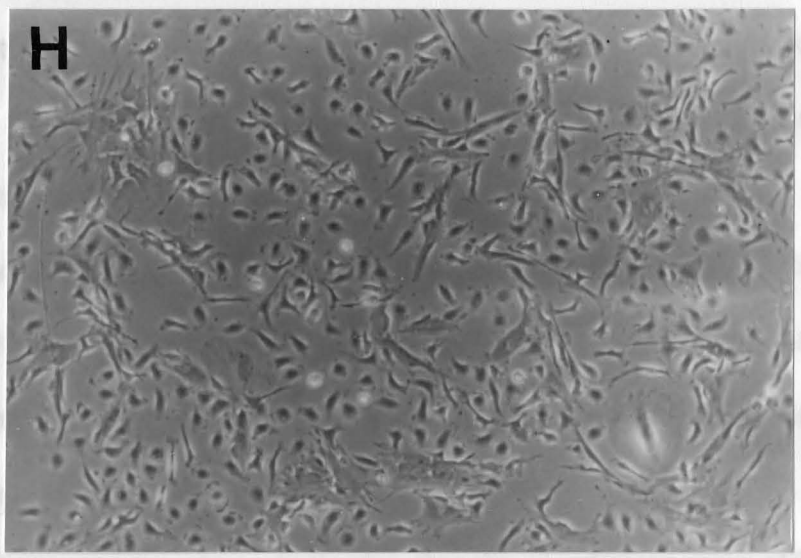

A
B
C

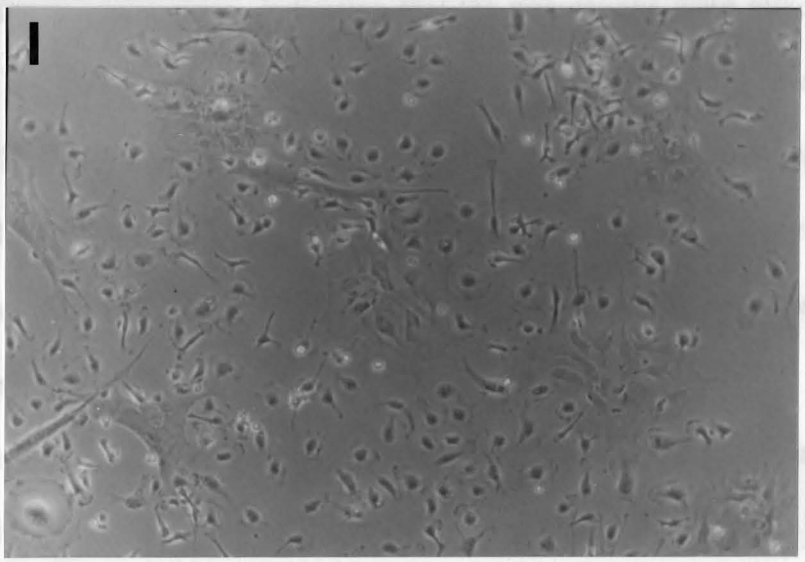

D


E


F


G


H


I

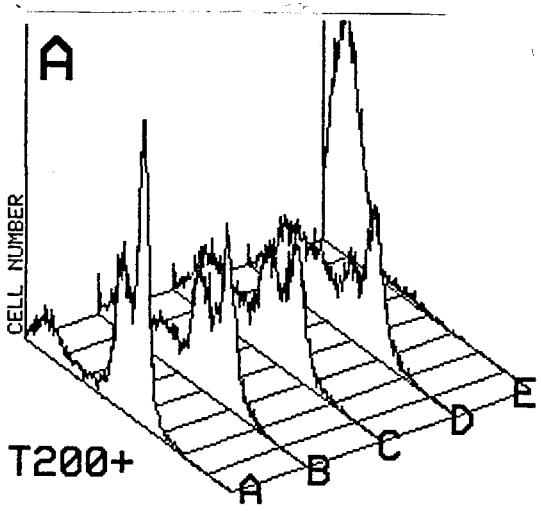
sufficient adherent layer, aggregates appeared to be an enriched source of lymphocyte-supporting stromal cells.

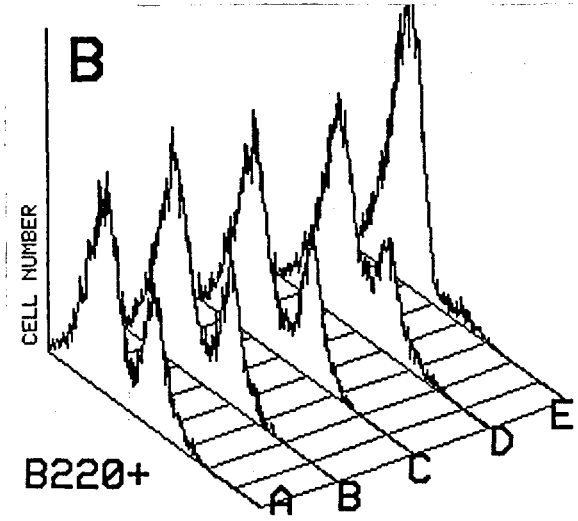
Phenotypic analysis of hemopoietic cells present in aggregates. Because long term lymphopoiesis forms from aggregates, progenitor cells as well as the appropriate stromal cells must be present. Stromal cell-progenitor cell contact is required for *in vitro* lymphopoiesis (112) and similar contacts have been reported *in vivo* (68). However, the precise stages of B-lymphocyte differentiation that are sequestered by stromal cells remains unknown. The apparently naturally-formed cell aggregates would seem to offer an avenue toward addressing this question. Thus, aggregates were dispersed using collagenase and the cells subjected to various phenotypic analyses, comparing these with unfractionated and deaggregated marrow suspensions.

Overall hemopoietic content of aggregates was assessed by FACS analysis using mAb M1/9 against mouse T200 (CD45) (171)(Fig. 7A) and by morphology after Jenner/Giemsa staining (Table 4). The frequency of $T200^+$ cells and their mean fluorescence intensity were similar in unfractioned, deaggregated, and dispersed aggregate marrow suspensions, indicating that the populations were alike in their total hemopoietic content. The T200 antigen seemed to be slightly degraded by this collagenase lot; however, the curves for unfractioned collagenase treated marrow, deaggregated marrow, and aggregates were all overlapping. By differential counting, little difference overall was seen between unfractioned marrow and dispersed aggregates, with the exception of eosinophils.

Because morphology alone cannot discriminate early B cell stages, a panel of well described B lineage markers was used to assess whether early B cells were more frequent

Figure 7. FACS profiles of bone marrow populations. In all figures A refers to untreated marrow, B to unfractioned collagenase treated marrow, C to deaggregated marrow, D to collagenase dispersed aggregates, and E to untreated marrow stained with only the secondary antibody reagent. A) T200 staining. B) B220 staining. C) BP1 staining. Note that panel C compares the fluorescence intensity of untreated marrow (shaded histogram) to that of dispersed aggregates (unshaded histogram). Histograms in panel C are gated on cells with forward and side scatter channels typical for lymphoid cells. All profiles represent 2 to 6 experiments for each antibody. Relative cell number is on the vertical axis and increasing fluorescence intensity is on the horizontal axis.


A
CELL NUMBER
T200+
A
B
C
D
E


B
CELL NUMBER
B220+
A
B
C
D
E

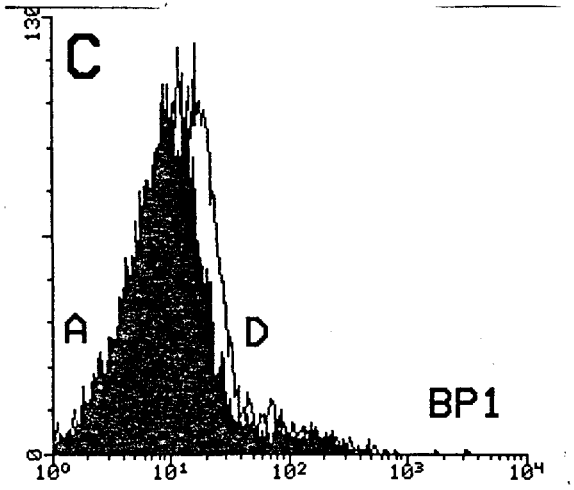

C
A
D
BP1
0
10⁰
10¹
10²
10³
10⁴

Table 4.--Morphology of Cells in Bone Marrow Populations[a]

	Untreated	Deaggregated[b]	Aggregates[b]
Lymphoid	22.3	29.6 ± 3.0[c]	24.1 ± 2.0
Granulocytic	39.0	48.6 ± 8.4	34.4 ± 4.7
Erythroid	34.6	18.6 ± 3.6	34.8 ± 7.6
Eosinophilic	2.8	3.3 ± 1.7	6.1 ± 0.9

[a] Cytocentrifuge preparations of each of the respective populations were stained with Jenner/Giemsa. [b] Dispersed with collagenase. [c] Average of three experiments ± S.D.

in the stromal cell containing aggregate fraction. First, the B lineage marker mAb 14.8 (anti-B220) (29) was used to determine the frequency of committed B lineage cells (Fig. 7B). Again, no differences were seen among the groups with respect to either the percentage of $B220^+$ cells or their mean fluorescence intensity. Next, the mAb BP1 (172) was used to examine the content of pre-B cells and newly formed B cells. FACS analysis with BP1 showed that there was a low level staining of all groups, which is characteristic of this antibody (data not shown). Aggregates, however, showed an increase in the frequency of $BP1^+$ cells as compared to whole marrow, suggesting an enrichment of immature B cells in aggregates (Fig. 7C). To further define this apparent enrichment two-color immunofluorescence microscopy for μ chain was performed to determine the numbers of pre-B ($c\mu+$) cells and B ($s\mu+$) cells in each population (Table 5)(32). The collagenase treatment had no effect on the ability to detect $s\mu+$ cells. The data demonstrate that deaggregated marrow was virtually identical to whole marrow with respect to its content of pre-B and B cells. Aggregates, however, contained a consistently higher frequency of $c\mu^+$ pre-B cells and lower frequency of B cells than whole marrow.

Hardy et al. have shown that pre-B cells prior to μ expression are responsive to IL-7 (33). IL-7 responsiveness can be assessed in a CFU-pre-B assay as described by Lee et al. (34). This assay was used to assess the number of clonable IL-7 responsive cells in aggregates (Table 6). Although the actual numbers of CFU-pre-B varied considerably from experiment to experiment, the data within each experiment indicated no selective enrichment or depletion of CFU-pre-B in any of the groups. It must be noted that this assay only enumerates those cells capable of responding to IL-7 alone.

Table 5.-- Percentage of B(sμ+) and pre-B(cμ+) Cells in Bone Marrow Populations[a]

	Untreated		Unfractioned[b]		Deaggregated[b]		Aggregates[b]	
	pre-B	B	pre-B	B	pre-B	B	pre-B	B
Expt. 1	14.9 ± 2.3	10.9 ± 3.1	16.7 ± 1.7	10.0 ± 1.9	16.0	16.4	23.1	7.6
Expt. 2	16.7 ± 0.3	8.7 ± 1.5	16.8 ± 3.3	6.1 ± 3.7	14.4 ± 0.4	6.4 ± 2.4	23.4 ± 1.7	3.0 ± 1.3
Expt. 3	14.0 ± 1.4	7.4 ± 0.8	14.8 ± 1.3	9.9 ± 0.7	11.7 ± 0.5	10.5 ± 0.9	20.7 ± 1.4	7.4 ± 1.7

[a] B and pre-B cell numbers were determined by staining cells in suspension with FITC conjugated anti-mouse μ to determine sμ+ cells. These cells were then spun onto cytocentrifuge spots, fixed in cold ethanol/acetic acid, and stained with TRITC conjugated anti-mouse μ to determine cμ+ cells. A minmum of 250 cells per slide were scored on a epifluorescence microscope. [b] Treated with collagenase. [c] Average of three slides ± S.D.

Table 6.--CFU-pre-B (IL-7) in Bone Marrow Populations[a]

	Untreated	Unfractioned[b]	Deaggregated[b]	Aggregates[b]
Experiment 1.	64.3 ± 3.3	55.5 ± 9.3	99.3 ± 19.0	29.7 ± 5.8
Experiment 2.	59.0 ± 9.2	72.8 ± 6.1	76.7 ± 26.6	64.8 ± 6.9
Experiment 3.	31.5 ± 26.1	37.8 ± 1.3	21.0 ± 4.1	28.3 ± 2.5

[a] 10^5 cells of each bone marrow population were plated in an IL-7 colony assay as described in *Materials and Methods*. Colonies >20 cells were counted after 6 days incubation. Results are presented as the mean ± S.D. of 4 individual plates. [b] Treated with collagenase.

Earlier B cell types also require stromal cell contact for proliferation (33) and are presumably not detected in the semi-solid agar assay.

The enzyme terminal deoxynucleotidyl transferase (TdT) may be the first identifiable marker of early B lineage cells and represents a means to measure early B lineage precursors that may not be responsive in the IL-7 colony assay. Table 7 shows that there was a consistent increase in the frequency of TdT+ cells in the aggregates and a consistent decrease in these cells in deaggregated marrow. This finding suggests that aggregates are enriched for very early B lineage cells that may be in close association with stromal cells *in vivo*.

Given that the earliest identifiable B cell precursors are more mitotically active than later stage cells (173,174) the proliferative activity of aggregate cells was examined. Table 8 shows that equivalent numbers of aggregate cells incorporated 2 to 4 times as much ^{3}H TdR as whole marrow or deaggregated marrow in an 18 hour pulse. Since the stromal cells that presumably provide signals for cell proliferation have been removed from deaggregated marrow, the possibility existed that the cells in that fraction exited the cell cycle and incorporated less radioactivity. To more definitively explore differences in the proliferative activity of aggregates from other marrow fractions, cell cycle analysis by propidium iodide staining of nuclear DNA (Table 8)(167) was performed. The results show a modest but consistent increase in the frequency of cells in $S+G_2M$ of the cell cycle in aggregates versus deaggregated marrow.

CONCLUSIONS

This work led to the following conclusions: 1) stromal cells are enriched in cellular aggregates that can be separated from marrow cell suspensions, 2) stromal cells

that can be identified in short-term culture constitute a very minor proportion of total marrow cells, 3) cellular aggregates are sufficient to form LTBMC-B cultures and must, therefore, contain both stromal cells and very primitive hemopoietic precursors, 4) short term culture of stromal cells is serum sensitive but relatively independent of serum concentration, extracellular matrix components, or initial plating density, 5) aggregates may preferentially include cells from the subendosteal marrow, 6) aggregates appear to represent areas of native marrow architecture, 7) there is little evidence for sequestering of middle and late stage B cell progenitors in the aggregates, and 8) aggregates are enriched in TdT^+ early B cells.

Table 7.--Frequency of TdT+ Cells in Bone Marrow Populations[a]

	Untreated	Unfractioned[b]	Deaggregated[b]	Aggregates[b]
Experiment 1.	1.5	1.4	N.D.[c]	3.9
Experiment 2.	1.3	1.4	0.8	3.1
Experiment 3.	N.D.	1.6	0.6	4.0

[a] TdT positive cells were determined by indirect immunofluorescence on fixed cytocentrifuge spots. A minimum of 600 cells were scored per slide. [b] Treated with collagenase. [c] Not Done.

Table 8.--Proliferative Activity of Marrow Fractions

		Untreated	Unfractioned[a]	Deaggregated[a]	Aggregates[a]
cpm of ^{3}H Tdr Incorporated[b]	Expt. 1	4411 ± 852	5275 ± 2370	7911 ± 1281	20937 ± 143
	Expt. 2	5980 ± 2009	3745 ± 4479	3175 ± 498	19154 ± 7155
%S + G_2M[c]	Expt. 1	22.7	21.2	22.6	26.6
	Expt. 2	22.0	23.8	24.1	28.0

[a] Treated with collagenase. [b] As determined by 18 hour pulse with ^{3}H Tdr. [c] As determined by analysis of propidium iodide staining of fixed cells on FACStar Plus.

Chapter III

ACTIVITY OF KIT LIGAND AND INTERLEUKIN-7 IN COMBINATION ON NORMAL BONE MARROW B LINEAGE CELLS

ABSTRACT

The production of B cells is regulated by soluble and cell contact signals presumably provided by bone marrow stromal cells. Among these is interleukin-7, a well characterized proliferative stimulus for a subset of pre-B cells. Kit ligand, a stromal cell derived cytokine with broad hematopoietic effects, has been reported to synergise with interleukin-7 to drive the proliferation and differentiation of $B220^-$ bone marrow cells into $B220^+$ B cell precursors in long term culture. A subsequent report has cast doubt on this result by showing that kit ligand and IL-7 were incapable of producing μ^+ pre-B cells after short term culture. Here, using the cell sorter to assure discrete separation of $B220^+$ and $B220^-$ cells followed by soft agar culture to prevent interaction with accessory cells, I demonstrate that the combination of kit ligand and IL-7 does not stimulate the expansion or differentiation of $B220^-$ lymphoid precursors but can act synergistically in the clonal proliferation of $B220^+$ cells. These experiments were begun in an attempt to develop an assay for $B220^-$ B cell precursors which could then be applied to aggregates. The results here confirm a role for kit ligand in B lymphopoiesis, supporting the rationale for examining it's expression in freshly isolated stromal cells.

INTRODUCTION

Having begun to dissect the stages of B cell development that are enriched in aggregates, a method to determine the frequency of the earliest B cell precursors was desirable. Such a method had not been reported, but work by McNiece and coworkers suggested that the combination of kit ligand (KL) and IL-7 could stimulate B cell formation from marrow that had been depleted of all identifiable committed B cells (38). Therefore, experiments based on the results of McNiece *et al.* were undertaken to develop such a quantitative assay. As well, firm evidence for a role for KL in B lymphopoiesis would make this an important cytokine to study in freshly enriched stromal cells.

A number of growth factors can influence B lymphocyte development. Principal among these is interleukin-7 (IL-7)(159). IL-7 acts as a proliferative stimulus on a subset of pre-B cells that express μ heavy chain in the cytoplasm ($c\mu$). This is the basis of a clonal soft agar culture assay that can be used to quantitate the frequency of pre-B cells (34). Hardy and colleagues described $B220^+$ cells prior to μ expression that are dependent on both IL-7 and contact with a stromal cell line (33). As well, $c\mu^+$ pre-B cells develop from $B220^-$ forebears in response to a factor(s) from another stromal cell line (143).

KL is a stromal cell derived cytokine that synergizes with other cytokines to stimulate multiple lineages of blood cells, most prominently the erythroid and myeloid lineages (52). McNiece and coworkers reported that KL in combination with IL-7 in liquid culture causes the proliferation and differentiation of $B220^-$ cells to become $B220^+$

cells (38). Indeed, stromal cell derived KL seems a likely candidate for promoting differentiation of early B cells, a subset of which expresses the KL receptor (c-kit)(162).

While it is possible that KL stimulates $B220^-$ pro-B cells to proliferate and differentiate, it is difficult to reconcile this with previous *in vivo* data suggesting that B lymphopoiesis can proceed even if c-kit is inhibited (162), and B cell development appears normal in adult *Sl/Sl*^{d} and *W/W*^{v} mice (175,176). Using a short term culture assay, Billips *et al.* argue against a role for KL in differentiation of $B220^-$ B cell precursors to become $c\mu+$ pre-B cells (39). These authors contend that the conclusions of McNiece *et al.* are incorrect due to insufficient purity of the $B220^-$ cells. Yet, the observations of Billips and colleagues are limited to the expression of μ protein; proliferation and expression of B220 were not examined on normal marrow B cell precursors. What remains, then, is doubt about the role of KL in B lymphocyte development. Here the FACS was used to obtain homogeneously pure populations of $B220^+$ and $B220^-$ cells from mouse bone marrow and their responses to KL, with and without IL-7, were examined in a classical soft agar colony assay. This system prevents any interaction with accessory cells that could influence B cell precursors and allows clear delineation of their response to these factors.

MATERIALS AND METHODS

Cell Preparation and Flow Cytometry. A fresh marrow cell suspension was washed once and resuspended at 2 x 10^7 cells per ml in Hank's balanced salt solution with 0.1% BSA and monoclonal antibody 14.8 (29)(anti-B220, ATCC, Rockville, MD). After 30 minutes incubation on ice the cells were washed, resuspended, and stained with FITC-

labelled mouse-anti-rat Ig (heavy and light chains, Jackson Labs, West Grove, PA). Cells were then washed again; fluorescence positive and negative cells were sorted using a Becton Dickinson FACStar PLUS (Mountain View, CA).

Colony Forming Unit (CFU) Assays. Recombinant IL-7 (Sterling Drug, Malvern, PA) was used at 1000U/ml (specific activity 4.7 x 10^7 U/mg) and recombinant rat KL (lot # R500-1, Amgen, Thousand Oaks, CA) was used at 100ng/ml. These concentrations resulted in maximal colony formation in titration experiments.

Cell Phenotyping. Colonies were removed from the soft agar cultures using a 5μl pipetman, being careful to remove as little agar as possible. Approximately 5 x 10^4 pooled cells were cytocentrifuged onto glass slides and stained with Jenner/Giemsa. The content of lymphoid, myeloid, or undifferentiated cells was determined by microscopic examination. Separate slides were stained with mAb 14.8 followed by FITC-conjugated mouse-anti-rat Ig (Jackson Labs). Other slides were fixed in ethanol/acetic acid at 4°C and stained with TRITC-conjugated goat-anti-mouse μ (Jackson Labs). The cells were then scored for $B220^+$ or μ^+ cells using an epifluorescence microscope.

Liquid Culture of $B220^-$ Cells. $B220^-$ cells were sorted directly into 24 well plates (Corning, Corning, NY) containing 1 ml of RPMI 1640 supplemented with 20% FCS and growth factors as described above. The final cell concentration was 2 x 10^5/ml. Cultures were incubated at 37°C in 7.5% CO_2. At 7 days 0.5 ml of medium was replaced with fresh medium containing growth factors and any cells removed were returned to the cultures.

RESULTS AND DISCUSSION

To determine if KL plus IL-7 caused $B220^-$ bone marrow cells to become pre-B cells, it was necessary to separate $B220^+$ and $B220^-$ cells. FACS sorting was used to do this because it can effectively separate cells expressing low levels of a given cell surface molecule. This was an important consideration because the earliest B lineage cells express low levels of B220 (33). These $B220^{dim}$ cells may respond to KL plus IL-7, yet are already committed to development within the B lineage. These would also be the most likely contaminants of $B220^-$ cells selected by panning. To critically determine the phenotype of cells responding to KL plus IL-7 it was important to include $B220^{dim}$ cells in the $B220^+$ fraction. Bone marrow was prepared and stained for B220 expression as described. An aliquot of the stained cell suspension was set aside for use as the unsorted marrow control. Cells were sorted according to the gates shown in Figure 8A. Post-sort analyses of the $B220^-$ and $B220^+$ populations are given in Figure 8B and C, respectively. After sorting, cells were cultured in soft agar with IL-7, KL, KL plus IL-7, or no added growth factors.

The results of three independent experiments are shown in Figure 9. From unsorted marrow, IL-7 produced colonies composed of μ^+ lymphoid cells, as described previously (34 and Table 9). KL alone resulted in variable numbers of colonies but few cells were lymphoid (Table 9, A. Varas, unpublished observations). The numbers of colonies resulting from KL plus IL-7 were additive. Many colonies formed from KL plus IL-7 were larger than those formed in the presence of IL-7 alone, both in unsorted and $B220^+$ fractions. Cells responding to the combination of KL and IL-7 were a mixture of

lymphoid and myeloid lineages.

As expected (34), all responsiveness to IL-7 alone was found among the $B220^+$ cells. In the first and third experiments, the addition of KL did not lead to increased colony formation by $B220^+$ marrow. In the second experiment, the addition of KL led to an increase in colony formation ($p > .005$). This may reflect the variability previously noted in the pre-B colony assay using BALB/c marrow (Chapter II). Nonetheless, IL-7, with or without KL, stimulated colonies composed almost entirely of lymphoid cells, the majority of which expressed μ (Table 9).

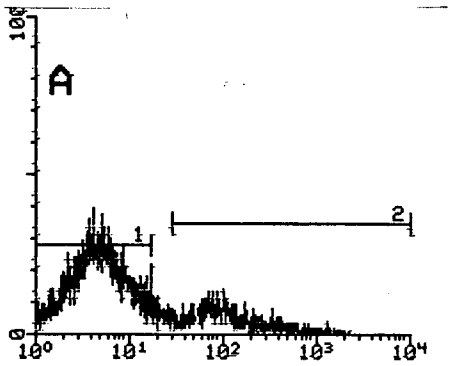
Conversely, all responsiveness to KL alone was confined to $B220^-$ marrow. In all experiments KL plus IL-7 yielded only as many colonies as KL alone from $B220^-$ marrow. KL produced colonies composed of myeloid and undifferentiated cells, regardless of the presence of IL-7. Therefore, the $B220^-$ cells, without the help of accessory cells or other factors, appear unresponsive to IL-7. The colonies in experiment 2 could not be phenotyped due to the poor colony response to KL.

The proliferation of B cell progenitors in liquid culture with IL-7, KL, or KL plus IL-7, as described by McNiece *et al.* (38) was also tested. $B220^-$ marrow cells were directly sorted into 24 well plates containing the growth factors. Even with media replacement at 7 days, all cultures declined and by 14 days no viable cells remained. It is possible that the cell proliferation reported in their study was supported in part by stromal cells present in the $B220^-$ marrow. No adherent cells were present in my cultures, probably because stromal cells are largely bound in cell aggregates which would not pass through the 70μm sort nozzle (Chapter II).

CONCLUSIONS

This work led to the conclusion that KL acts with IL-7 on cells already expressing B220 and committed to development as B lymphocytes. The combination of KL and IL-7 in culture, therefore, does not constitute an assay system for $B220^-$ pro-B lymphocytes, as would be predicted by the data of McNiece and coworkers (38). The use of soft agar culture prevents contact with accessory cells that could influence differentiation of primitive B cell predecessors. As suggested by Billips *et al.* (39), such contacts cannot be replaced by the addition of soluble KL.

Figure 8. A) Fluorescence profile of normal mouse bone marrow stained for B220 expression in experiment 1. Relative cell number is on the Y axis and log fluorescence intensity is on the X axis. Region 1 indicates sorting gates used to separate $B220^-$ cells. Region 2 indicates the sorting gates used to separate $B220^+$ cells. B) Post-sort analysis of $B220^-$ cells, purity >99%. C) Post-sort analysis of $B220^+$ cells, purity >93%. In experiment 2 the purity of both populations was 98%. In experiment 3 the $B220^+$ cells were >93% pure and the $B220^-$ cells >99% pure.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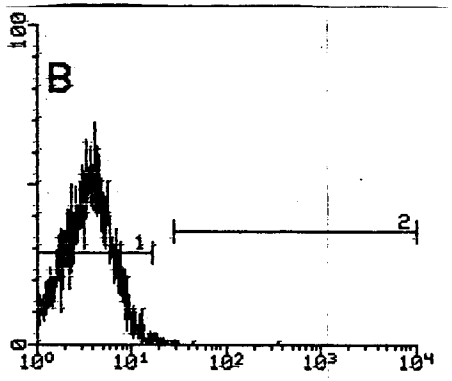
0
1
2
10^0
10^1
10^2
10^3
10^4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10^3
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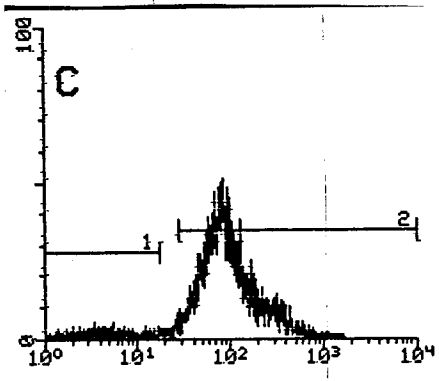
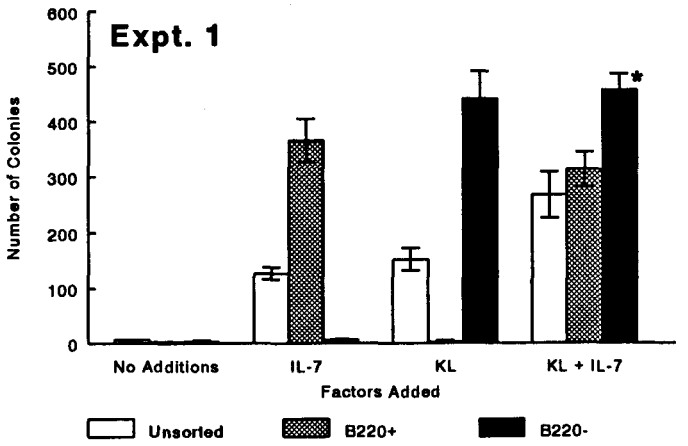
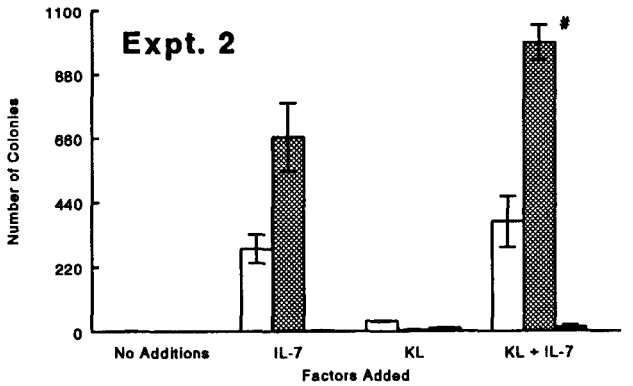

C
100
0
1
2
10^0
10^1
10^2
10^3
10^4

Figure 9. Colony formation by unsorted, $B220^+$, and $B220^-$ marrow cells in response to KL, IL-7, or KL+IL-7. All bars represent the mean $\pm$ SD of at least three plates except * which denotes the mean $\pm$ SD of two plates. # denotes a significant increase in colony response ($p > 0.005$) over that seen with IL-7 alone.


Expt. 1
Number of Colonies
600
500
400
300
200
100
0
*
No Additions
IL-7
KL
KL + IL-7
Factors Added
Unsorted
B220+
B220-


Expt. 2
Number of Colonies
1100
880
660
440
220
0
#
No Additions
IL-7
KL
KL + IL-7
Factors Added

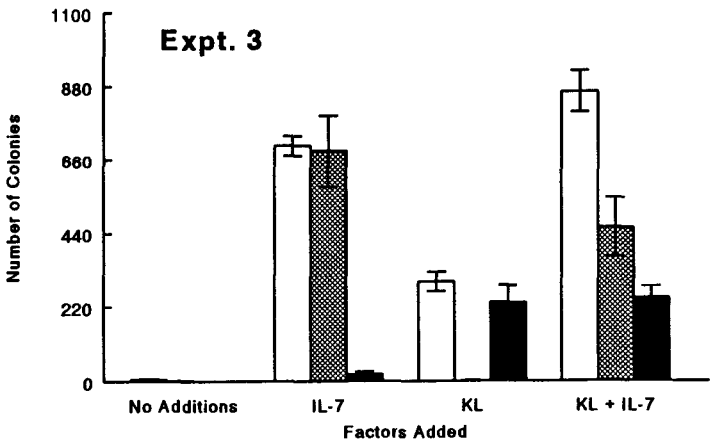

Expt. 3
Number of Colonies
1100
880
660
440
220
0
No Additions
IL-7
KL
KL + IL-7
Factors Added

Table 9.--Phenotype of Cells in Colonies in Experiments 1 and 3

		Percent of Total[a]				
Cells	Condition	Lymphoid[b]	Myeloid[b]	Blast[b]	μ+[c]	B220+[d]
Unsorted	IL-7	86; 97[e]	8; 3	6; 0	86; 93	ND; 85
	KL	4; 2	72; 85	24; 13	6; 3	ND; 4
	KL + IL-7	64; 68	23; 29	13; 3	55; 45	ND; 61
B220+	IL-7	90; 95	2; 5	8; 0	76; 93	ND; 94
	KL + IL-7	88; 97	4; 3	9; 0	67; 87	ND; 99
B220-	KL	5; 3	77; 97	17; 0	0[f]; 0	ND; 0
	KL + IL-7	4; 11	74; 79	21; 11	2; 6	ND; 0

[a] A minimum of 100 cells were scored per slide except where noted. B220+ with KL and B220- with IL-7 resulted in too few cells to analyze. ND; Not Done. [b] Assessed by morphology after Jenner/Giemsa stain. Blasts column denotes cells with undifferentiated morphology. [c] Stained for μ immunofluorescence without distinguishing cμ from sμ cells. [d] Determined by indirect immunofluorescence staining with mAb 14.8. [e] Analyses of colonies from experiments 1; 3. [f] Only 15 cells seen on this slide.

Chapter IV

NATIVE ASSOCIATIONS OF EARLY HEMOPOIETIC STEM CELLS AND STROMAL CELLS ISOLATED IN BONE MARROW CELL AGGREGATES, INCLUDING DEVELOPMENT OF A QUANTITATIVE ASSAY FOR 'pro-B' CELLS

ABSTRACT

In suspensions of murine bone marrow many stromal cells are tightly entwined with hemopoietic cells. These cellular aggregations appear to exist normally within the marrow. Previous studies documented that lymphocytes and stem cells adhered to stromal cells via vascular cell adhesion molecule 1 (VCAM1). Injection of anti-VCAM1 antibody into mice disrupts the aggregates, demonstrating the importance of VCAM1 in the adhesion between stromal cells and hemopoietic cells *in vivo*. Early hemopoietic stem cells were shown to be enriched in aggregates by using a limiting-dilution culture assay. Myeloid progenitors responsive to WEHI-3CM in combination with kit ligand and $B220^-$ B cell progenitors responsive to insulin-like growth factor-1 (IGF-1) in combination with interleukin-7 (IL-7) are not enriched. These experiments further my characterization of the stromal cell-enriched aggregates.

INTRODUCTION

In initial attempts to isolate stromal cells from fresh marrow, it was found that a large portion of bone marrow stromal cells are physically entwined with hemopoietic cells, forming cellular aggregates in marrow cell suspensions (Chapter II). Stromal cells within these aggregates are highly reticular, as manifest by staining for two stromal cell associated molecules, alkaline phosphatase and VCAM1 (Chapter II,67,113). These stromal cell reticular processes interdigitate with numerous hemopoietic cells in much the same way as is seen in marrow sections (68,113). Thus, the aggregates appear to be naturally-formed and reflect normal cell associations in the marrow. VCAM1 is important for stromal cell/hemopoietic cell adhesion *in vitro* (113). Although it is expressed on stromal cells within native marrow, its function is as yet untested *in vivo*. The presence of VCAM1$^+$ stromal cells entwined with hemopoietic cells in aggregates represents a unique way to examine the use of VCAM1 mediated adhesion in normal bone marrow.

Aggregates are enriched in certain early B cell precursors and are capable of forming LTBMC-B from reduced numbers of initiating cells (Chapter II). Because lymphopoiesis initiates from Thy1^{lo} stem cells (177), the latter observation suggests that these may also be enriched within the aggregates. Sequestering of specific differentiative steps of hemopoiesis within aggregates may imply the necessity of stromal cell contact for that stage, enabling dissection of stromal cell dependent phases of development. In the present studies examined the content of early hemopoietic stem cells as well as early precursors of both the lymphoid and myeloid lineages within bone marrow cell

aggregates.

MATERIALS AND METHODS

Collagenase Dispersion of Aggregates. A cocktail of 0.2% collagenase (grade CLS 3 lot # FOD 671, Worthington, Freehold, NJ), 0.1% hyaluronidase (grade HSE, Worthington) and DNase I (Worthington) in RPMI + 10% FBS was used to digest aggregates into single cells. Cells were digested in this cocktail for 1.5 to 2 hr at 37°C with 70rpm agitation on an orbital shaker. After digestion, cells were washed twice in cold RPMI 1640 with 10% FBS before subsequent manipulations.

Anti-VCAM1 Treatment In Vivo. 50μg M/K2 (113)(anti-VCAM1) in PBS was injected *i.v.* into each mouse. In parallel, mice received 50μg rat IgG (Sigma, St. Louis, MO) in PBS or PBS alone. The animals were sacrificed 15 or 30 minutes after injection, the femurs were removed, and aggregates were isolated as described above. Aggregates were dispersed in 0.2% collagenase for 1.5 hr. and the viable cells counted and compared to the number of cells present per femur in the marrow before separation of the aggregates and collagenase treatment. Each animal was analyzed individually; cells from both femurs were pooled. Data on untreated bone marrow was taken from other representative experiments in the notebook. Statistical analysis was by t-test.

Long Term Bone Marrow Culture. LTBMC-B cultures were performed as described (Chapter II). The medium used for LTBMC-B contained serum lot 11112034 (Hyclone, Logan UT). Medium for LTBMC-M was Fisher's medium supplemented with 20% selected FBS (Lot 11111087, Hyclone), 2mM L-glutamine, 50 U/ml penicillin, and 50μg/ml streptomycin, and 10^{-6}M hydrocortisone. LTBMC-M were maintained at 33°C

at 5.0% CO_2 with medium replenishment once a week. Hemopoiesis was maintained in these cultures for at least seven weeks. The nonadherent cells were removed from LTBMC-M at 5 weeks and found to be predominantly neutrophils by morphological examination.

Cell Sorting. Unfractioned collagenase treated marrow or collagenase dispersed aggregate cells were washed and $B220^+$ and $^-$ cells sorted as previously described (Chapter III).

Colony-Forming Unit (CFU) Assays. Following collagenase digestion and/or FACS sorting, cells were placed in colony-forming unit assays as described previously (Chapter II and III). WEHI-3 cell conditioned medium, recombinant kit ligand (KL, 100ng/ml)(Amgen, Thousand Oaks, CA), Interleukin-7 (IL-7, 1000U/ml)(Sterling Drug, Malvern PA, or Biosource, Camarillo, CA), recombinant Insulin-like Growth Factor-1 (IGF-1, 50ng/ml)(Biosource) were added at concentrations that exhibited maximal colony formation (previously determined).

Limiting Dilution Analysis of Hematopoietic Precursors. BMS2 stromal cell line (132,134) or stromal cells removed from LTBMC-B were plated into 96 well plates (BMS2 10^4/well, LTBMC-B $1.5x10^4$/well) and allowed to grow overnight. BMS2 layers were irradiated with 2500 rad and LTBMC-B stromal cells were irradiated with 1000rad from a ^{137}Cs source. This served to halt proliferation of BMS2 cells and prevent the appearance of lymphoid cobblestones still associated with the LTBMC-B stromal cells. The medium (LTBMC-M) was replaced 24 hr. after irradiation. After a further 24 hr. dilutions of unfractioned marrow, deaggregated marrow, or dispersed aggregates that had

been identically treated in collagenase, hyaluronidase, and DNase were added. At least six dilutions steps differing by a factor of 2 to 5, i.e. $8x10^4$, $4x10^4$, $1x10^4$, $5x10^3$, $1x10^3$, $5x10^2$, were used with 20 replicate wells per step. Cultures were incubated under LTBMC-M conditions. At weekly intervals wells were scanned for the appearance of foci containing at least 15 hemopoietic cells attached to or beneath the stromal cells (cobblestone areas). Analysis of limiting dilution data was performed as described (178,179). Linear regression analysis of this data yielded a straight line passing near the origin indicating that a single, limiting cell type was being detected as described (178,179). All data presented had a correlation coefficient (r) of 0.93 or greater. The frequency of cobblestone forming cells was calculated by Poisson statistics at 37% of negative wells, using the linear regression equations derived.

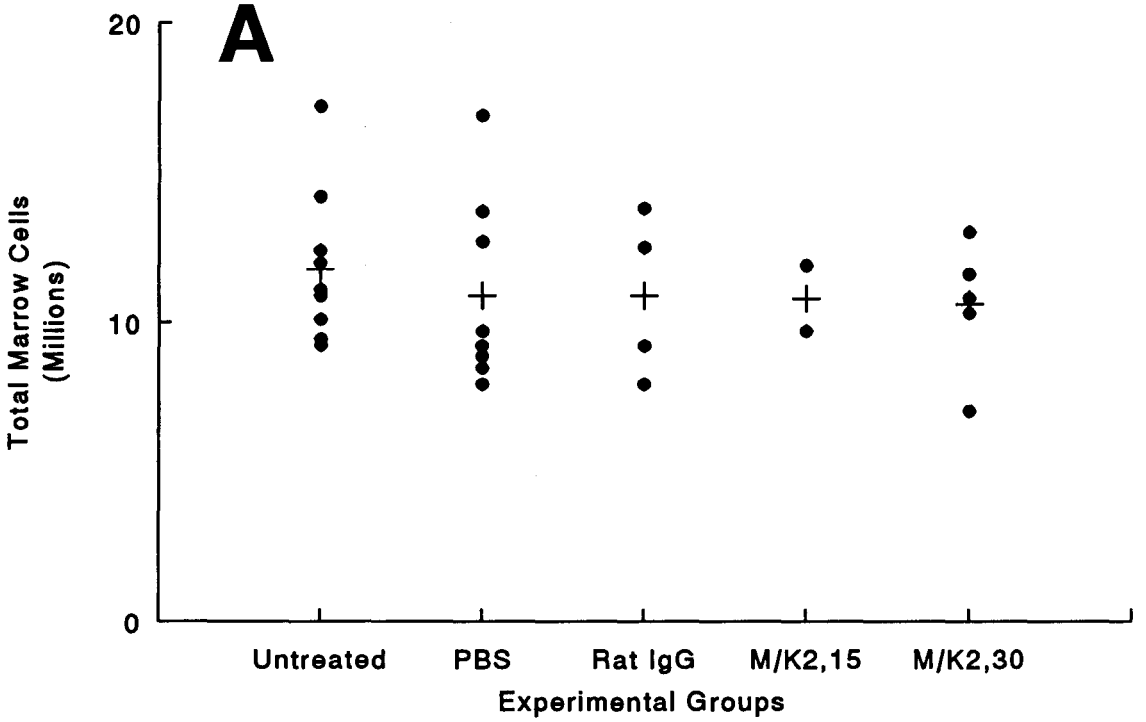
RESULTS

Evidence that marrow cellular aggregates reflect normal stromal/hemopoietic interactions. Many bone marrow stromal cells are found enmeshed in cellular aggregates (Chapter II). The staining pattern with two stromal cell markers, VCAM1 and alkaline phosphatase, retains the architecture seen in marrow sections (Chapter II,67,113). On this basis it has been suggested that aggregates are naturally-formed and reflect native hemopoietic-stromal cell contacts. VCAM1, expressed on stromal cells, and its ligand, VLA-4, expressed on hemopoietic cells, are involved in the adhesion of hemopoietic cells to stromal cells in long term culture and antibodies to either disrupts lymphopoiesis in LTBMC (113,116). If the *in vitro* data truly reflects the function of VCAM1 and VLA4 *in vivo*, then it should be possible to disrupt aggregates with antibodies to these proteins.

By visual inspection it appeared that anti-VCAM1 antibody, particularly in combination with anti-CD44 antibody, led to partial disruption of the aggregates in vitro. However, equal number and sized aggregates could not be placed into replicate tubes to test for dispersion by antibody; hence, different numbers of cells released might be due to more aggregates or smaller aggregates in a sample, not to better dispersion by antibody. Therefore it was not possible to quantify aggregate dispersion in vitro. Since the percentage of cells recovered in aggregates has been uniform over a number of experiments (Chapter II), it is reasonable that inhibition of molecules involved in the adhesion between stromal and hemopoietic cells *in vivo* would be reflected in a decrease in the fraction of cells present in aggregates. This would represent a quantitative way to look for disruption of the aggregates. In addition, the constant blood flow through the marrow would ensure that stromal cells were bathed in antibody whereas settling of aggregates during *in vitro* disruption experiments likely prevented them from being uniformly exposed to antibody.

Therefore, mAb M/K2 (113) was injected *i.v.* into mice to see if this treatment caused disruption of the aggregates. A dose 50μg of antibody per animal was chosen because that amount saturates cell surface VCAM1 in the marrow within minutes after injection and avoided modulation of the antigen from the cell surface (147). Figure 10A shows that there was no alteration in total marrow cellularity in anti-VCAM1-treated animals, indicating that there was no overt cell lysis or emigration from marrow caused by the antibody within 15 or 30 minutes following injection. However, the number of cells in aggregates was reduced significantly ($p > 0.025$) by treatment with anti-VCAM1

Figure 10. Effects of anti-VCAM1 antibody M/K2 on bone marrow cell aggregates. A) Marrow cellularity in each experimental group. Each dot represents one animal, the + denotes the average for each group. There was no significant difference between any of the groups. B) Percentage of total marrow present in aggregates. Treatments with M/K2 for 15 and 30 minutes were significantly different ($p > 0.025$) from control groups.


A
20
10
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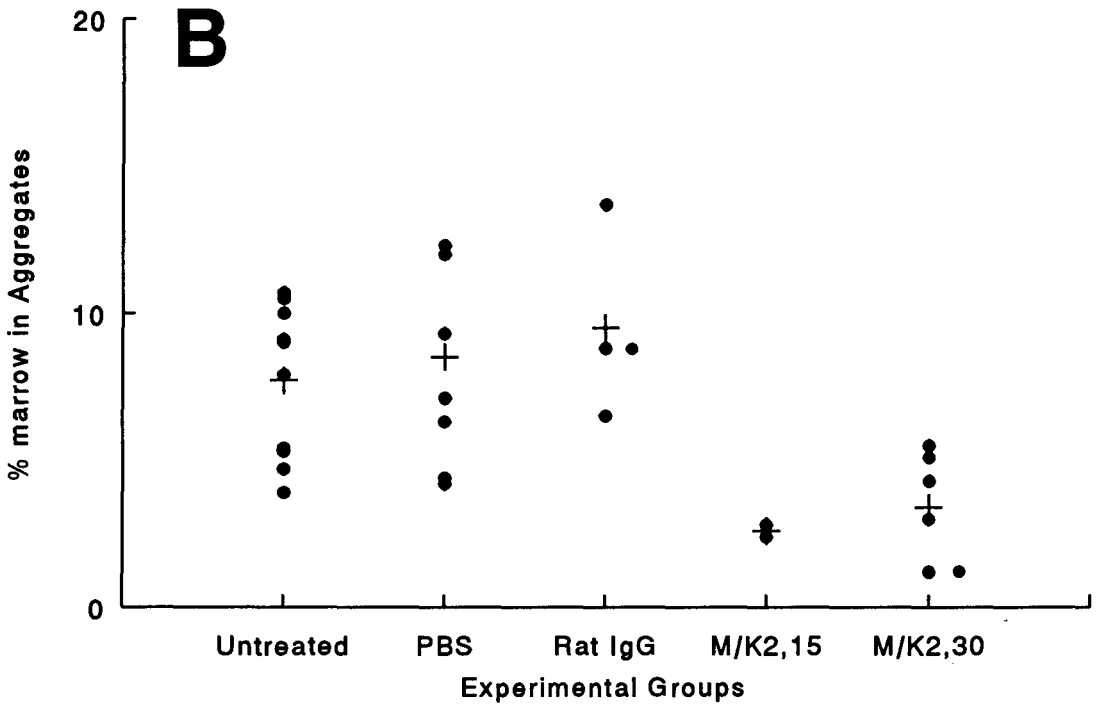
Total Marrow Cells
(Millions)
Untreated
PBS
Rat IgG
M/K2,15
M/K2,30
Experimental Groups


B
20
10
0
% marrow in Aggregates
Untreated
PBS
Rat IgG
M/K2,15
M/K2,30
Experimental Groups

antibody at either time point as compared to control animals (Figure 10B). The aggregates that remained contained culturable stromal cells at a frequency identical to that in control animals (data not shown), so it is unlikely that the disruption observed was due to lysis of stromal cells. This result strengthens the argument that the aggregates reflect typical cellular interactions occurring between stromal cells and hemopoietic cells in normal animals. This data also implicates VCAM1 in the adhesion of these cells in aggregates.

Frequency of early hemopoietic stem cells in aggregates. Highly enriched stem cells and B cell precursors use VLA4 to bind to VCAM1 on cultured stromal cells (118,119). VCAM1 expressing stromal cells are enriched in aggregates (Chapter II), therefore it seemed likely that hemopoietic stem cells would also be sequestered there. LTBMC-M cultures established readily from at least 50% fewer aggregate cells (data not shown) than were required of unfractioned marrow, demonstrating the presence of hemopoietic stem cells within the aggregates.

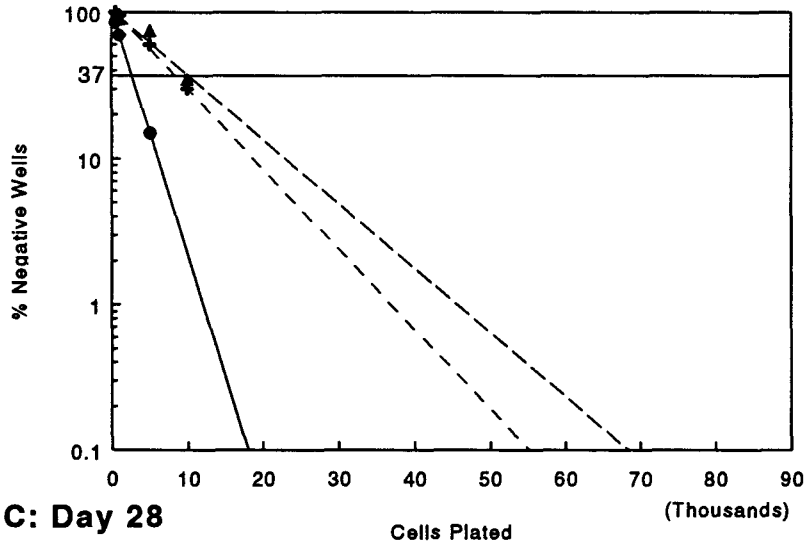
To address whether stem cells were indeed more frequent in the aggregates than in unfractioned marrow, the cobblestone-area-forming cell (CAFC) assay, a limiting-dilution assay to derive the frequency of the earliest hemopoietic stem cells, was performed. Several authors have equated the appearance of cobblestone areas at given time points with the primitiveness and self-renewal capacity of the stem cell (25,26,27). Two types of irradiated feeder layers were used, the stromal cell line BMS2 (132,134) and primary adherent cells (stromal cells and macrophages) from LTBMC-B. Both of these have been shown to support the development of hemopoietic cells in LTBMC-M

conditions (131,132,134). Cells from unfractioned marrow, deaggregated marrow, or aggregates were identically treated with collagenase/hyaluronidase and seeded onto feeder layers at various dilutions and the presence or absence of cobblestone areas within a well was scored at 7, 14, 21, and 28 days after seeding in LTBMC-M conditions. The results are presented as semi-log plots of the percent of wells without cobblestone areas. A linear regression line calculated from the data passes very close to the origin, indicating that a single, limiting cell type is being detected, in agreement with previous investigations (25,26,178,179). The point at which this line intersects 37% negative wells is the frequency of that stem cell type in the fraction examined. The data show that aggregates had a 3 to 4 fold higher frequency of CAFC than unfractioned collagenase treated marrow at all days tested (Figure 11, Table 10). Deaggregated marrow, on the other hand, usually contained CAFC only in proportion to its relative cellularity and the frequency of CAFC in deaggregated collagenase treated marrow was not significantly different from that for unfractioned collagenase treated marrow. Hence, aggregates contained 20-25% of all CAFC per femur while comprising only 6-7% of marrow cells. This demonstrated that early stem cells are sequestered in the aggregates, presumably in close association with stromal cells.

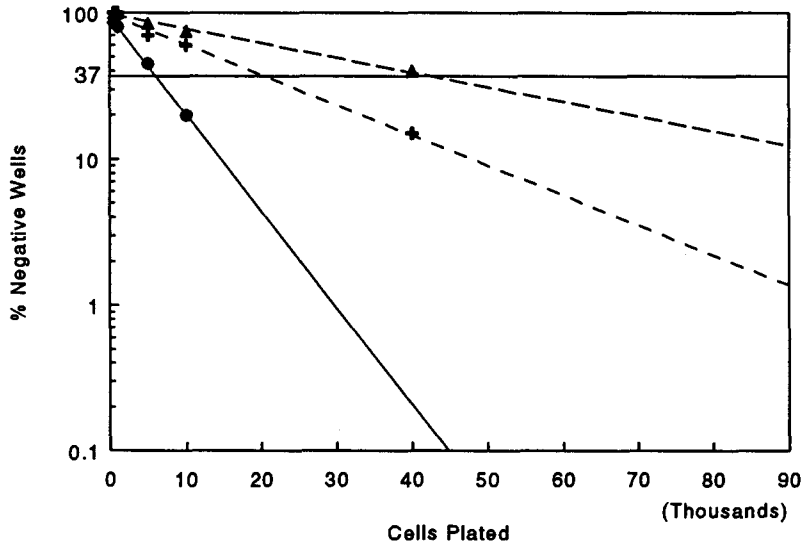
Content of clonogenic myeloid progenitors. Cells capable of forming cobblestone areas at day 28 have been correlated with marrow repopulating ability whereas later, more restricted cells are capable of forming splenic colonies (CFU-S) in lethally irradiated animals (25). Bone marrow progenitor cells highly enriched for CFU-S have been reported to proliferate in culture in response to IL-3 and kit ligand (KL)(180). Colony

Figure 11. Limiting dilution analysis of early hemopoietic stem cells. Unfractioned marrow (+), deaggregated marrow (▲), and aggregates (●) were identically treated with collagenase and hyaluronidase and multiple dilutions were plated onto irradiated BMS2 stromal cells. A cobblestone area was counted as a focus of 15 or more hemopoietic cells attached to or beneath the stromal cells. Data are presented as linear regression lines with input cell number on the x-axis and percentage of negative wells (without cobblestone areas) on the y-axis. The line at 37% negative wells intercepts the lines at the frequency of the progenitor type being measured. The linear regression lines pass close to the origin, indicating that a single, limiting cell type is being measured. Correlation coefficients (r) for all data presented $\geq$ 0.93. A) Cobblestone area forming cells at day 7 of culture. B) CAFC at day 14. C) CAFC at day 28. This is the first of three experiments shown in Table 1.

A: Day 7



B: Day 14



C: Day 28

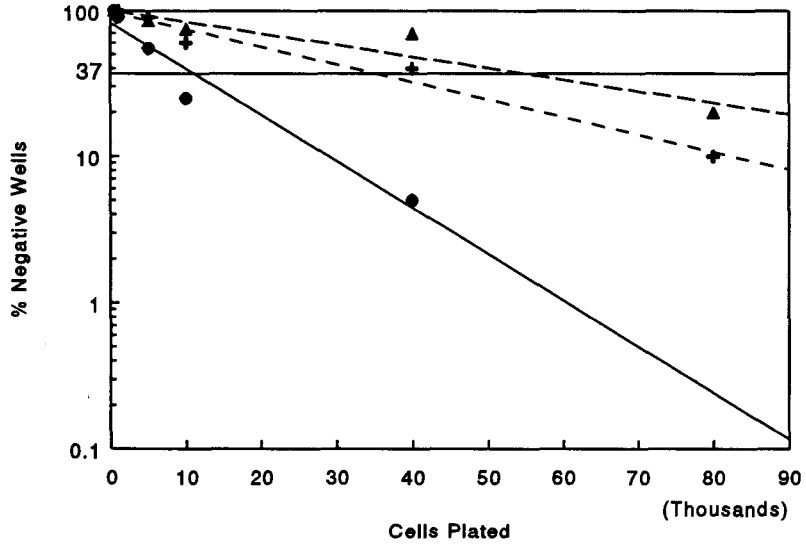
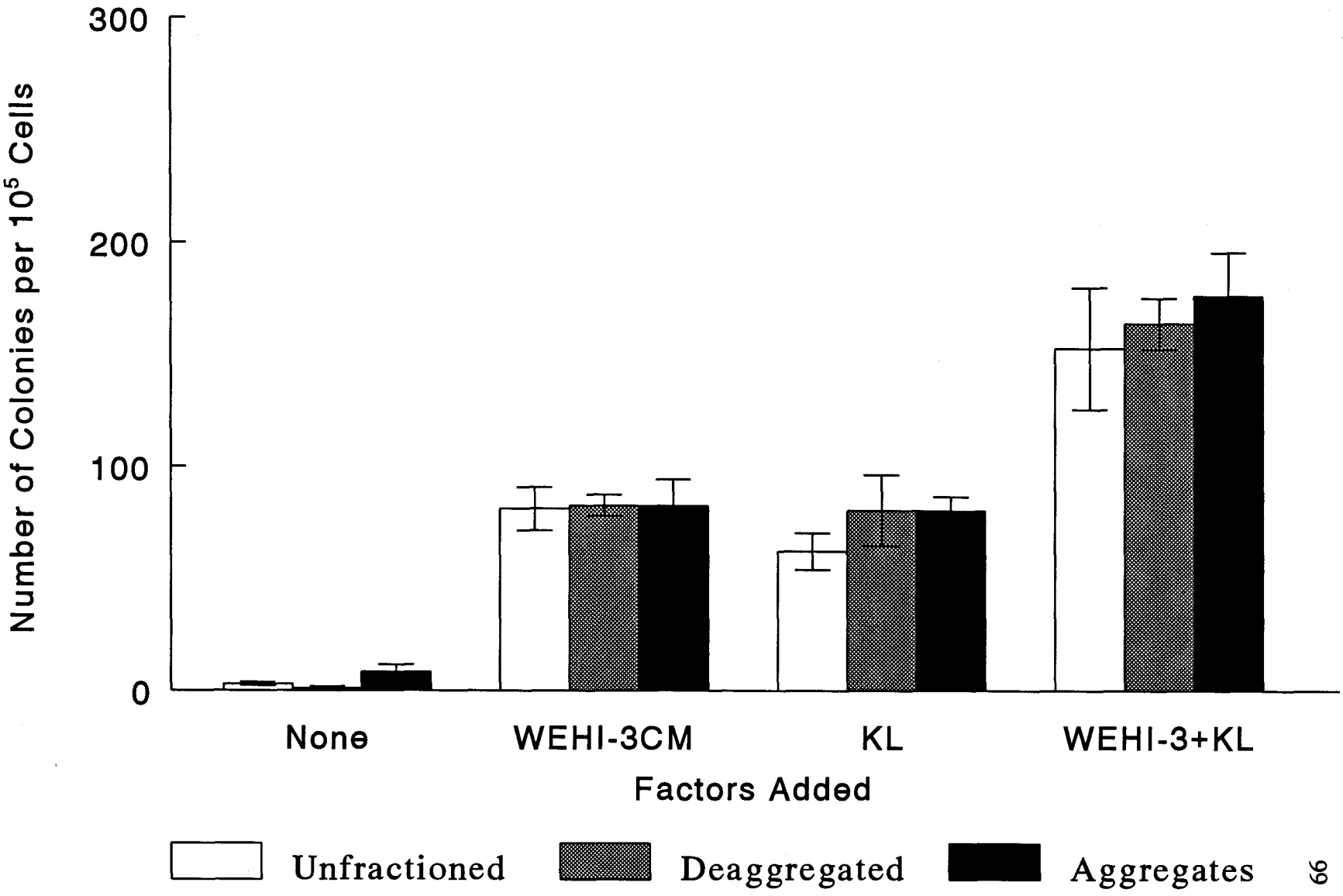


Table 10.--CAFC Frequency in Bone Marrow Fractions

			Cobblestone-Area Forming Cells			
			Day 7	Day 14	Day 21	Day 28
		Cells/femur (10^{-6})	1/Freq.[a]	1/Freq.	1/Freq.	1/Freq.
Unfrac[b]	I	10.9	8,496	20,621	41,334	35,565
	II	13.0	6,524	16,766	18,705	19,313
	III	17.8	10,244	13,838	N.I.[c]	N.I.
Deagg[b]	I	5.9	10,244	42,809	31,506	55,141
	II	6.5	3,343	9,629	15,232	41.465
	III	8.7	9,102	9,068	N.I.	N.I.
Agg[b]	I	0.4	2,657[d]	6,067	13,735	10,990
	II	1.2	1,817	5,293	13,114	14,019
	III	0.7	2,791	6,057	N.I.	N.I.

[a] Calculated from the intercept of the linear regression plots in Figure 2 with 37% negative wells. Correlation coefficients (r) for all data shown ≥ 0.93. [b] Treated with collagenase and hyaluronidase. [c] N.I. Not Interpretable. [d] Aggregates days 7 & 14 were significantly different from unfractioned marrow by t-test ($p > 0.01$). Deaggregated marrow was not significantly different. Too few samples to determine significance on day 21 & 28, but a similar trend is seen.

Figure 12. Clonogenic myeloid precursors in bone marrow fractions. Unfractioned, deaggregated, or aggregate cells were identically treated in collagenase and hyaluronidase and plated in soft agar culture with WEHI-3CM, SCF, or both and incubated for 6 days. Data shown are the mean $\pm$ S.D. of at least three plates.


300
200
100
0
Number of Colonies per 10^5 Cells
None
WEHI-3CM
KL
WEHI-3+KL
Factors Added
Unfractioned
Deaggregated
Aggregates

formation in the presence of IL-3 and KL was used to assess the frequency of immature factor dependent but stromal cell independent hemopoietic progenitors in marrow fractions (181,182). The results showed very little difference in the CFU-c frequency potentiated by WEHI3-CM (IL-3) between the marrow fractions; moreover, addition of rodent KL revealed no further differences (Figure 12). Some colony stimulating activity was noted from KL alone, as has been reported previously (181,182,Chapter III). Taken together, only the most primitive hemopoietic stem cells appeared to be sequestered within aggregates, whereas later, yet still immature myeloid restricted cells, capable of proliferation in response to growth factors alone, were not.

Content of lymphoid progenitors. Early B-cell precursors expressing TdT are 3-4 fold enriched in aggregates, whereas later stages in B-cell maturation (CFU-IL-7 and $sIgM^+$) are not enriched or are even somewhat depleted (Chapter II). Until recently assays to measure B-cell progenitors prior to B220(CD45RA) expression were lacking. Landreth *et al.* reported that the combination of IL-7 and Insulin-Like Growth Factor-1 (IGF-1) in short term liquid culture potentiates the differentiation of $B220(CD45RA)^-$ bone marrow cells to become $c\mu^+$ (40). Experiments were designed to see if this activity could be reproduced clonally in semi-solid culture, enabling quantitation of $B220(CD45RA)^-$ B cell precursors. $B220(CD45RA)^-$ bone marrow cells were purified by cell sorting and placed in soft agar culture with growth factors. As previously observed, IL-7 alone resulted in no colony formation by $B220(CD45RA)^-$ cells (Figure 13)(34,35,Chapter III). Unfractioned and $B220(CD45RA)^-$ marrow cells yielded a few colonies in response to IGF-1 alone, a majority of which were myeloid (Table 11, Figure

13 and 14). The combination of IL-7 and IGF-1, however, led to the formation of a small but consistent number of colonies (20-30 colonies per 10^5 cells) from B220(CD45RA)$^-$ marrow cells (Figure 13). These colonies were >95% lymphoid and μ^+ whether they originated from unfractioned or B220$^-$ marrow (Table 11, Figure 14). The addition of KL did not enhance the number of lymphoid colonies formed in the presence of IGF-1 and IL-7 (data not shown).

This system was then used to determine the frequency of B220(CD45RA)$^-$ B cell precursors in the bone marrow aggregates as compared to unfractioned marrow. B220$^-$ cells were isolated by FACS sorting from dispersed aggregates or unfractioned marrow and placed in soft agar culture containing growth factors. No differences were noted in the frequency of colonies formed in response to IGF-1 and IL-7 (Figure 15), implying that aggregates were not enriched in this early B cell progenitor. Although collagenase treatment has not affected cells in other functional assays (3), the possibility that the B220$^-$ precursors in aggregates are especially sensitive to collagenase treatment, which could alter their functional characteristics, cannot be completely excluded.

CONCLUSIONS

The results presented here extend previous work characterizing the hemopoietic cells entwined with stromal cells in the bone marrow. These experiments lead to the following conclusions: 1) the adhesion of cells in aggregates is mediated at least in part by the VCAM1 molecule, 2) aggregates are enriched in CAFC, a measure of primitive hemopoietic cells, 3) aggregates are not enriched in hemopoietic precursors responsive to IL-3, with or without KL, in culture, 4) the combination of IL-7 and IGF-1 stimulates

early B cell precursors in culture, and 5) aggregates are not enriched in B cell precursors that respond to IL-7 with IGF-1.

Figure 13. Colony formation in response to IGF-1, IL-7, or IGF-1 + IL-7. Unsorted or $B220^-$ cells were isolated by sorting from noncollagenase treated marrow and plated in soft agar culture with growth factors for 6 days. Data shown are the mean $\pm$ S.D. of at least three plates.

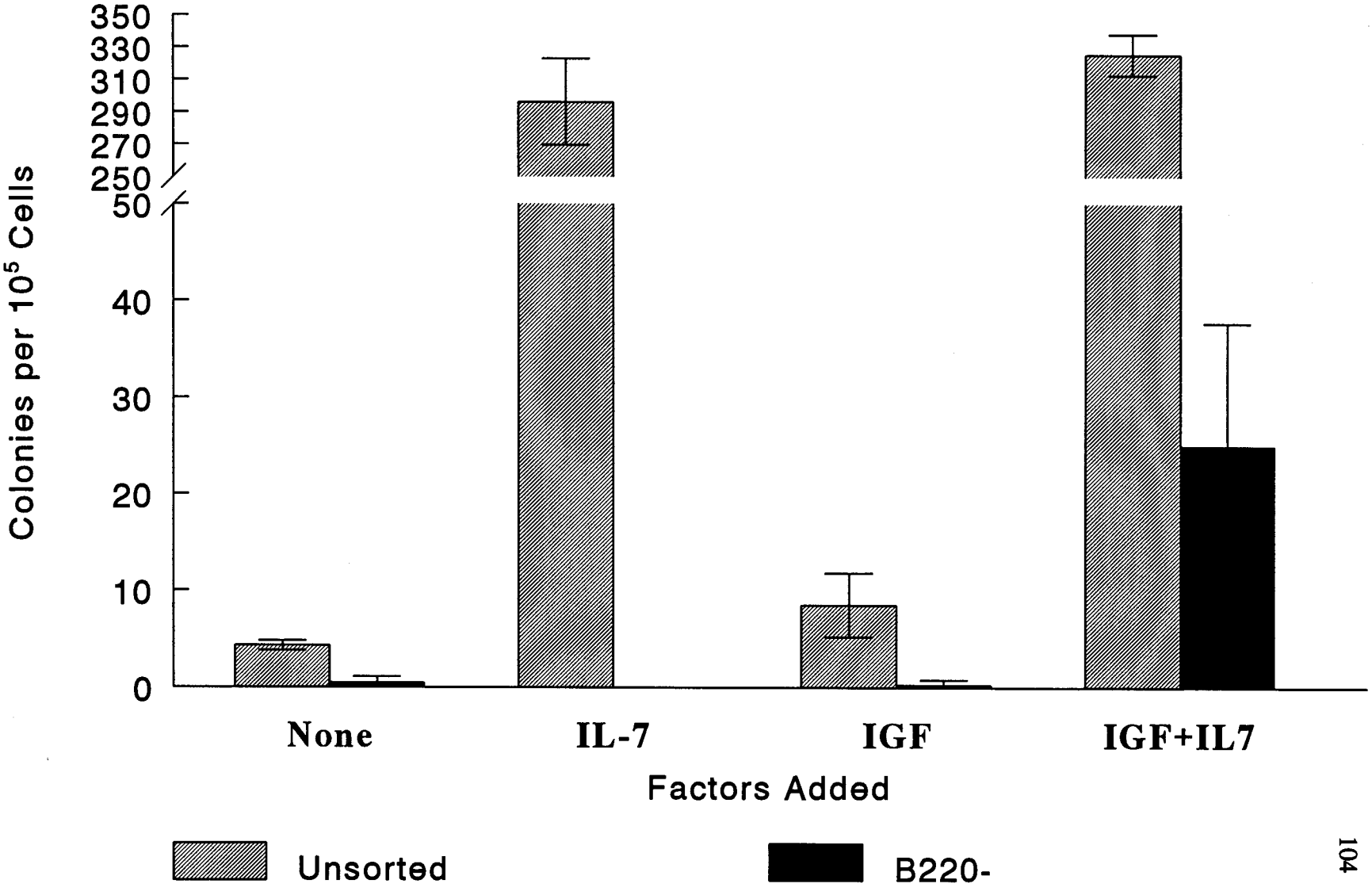

350
330
310
290
270
250
50
40
30
20
10
0
Colonies per 10^5 Cells
None
IL-7
IGF
IGF+IL7
Factors Added
Unsorted
B220-

Figure 14. Morphology of cells responding to growth factors. A) Unfractioned marrow with IGF-1 alone, B) Unfractioned marrow with IGF-1 + IL-7, and C) $B220^{-}$ marrow cells with IGF-1+IL-7.

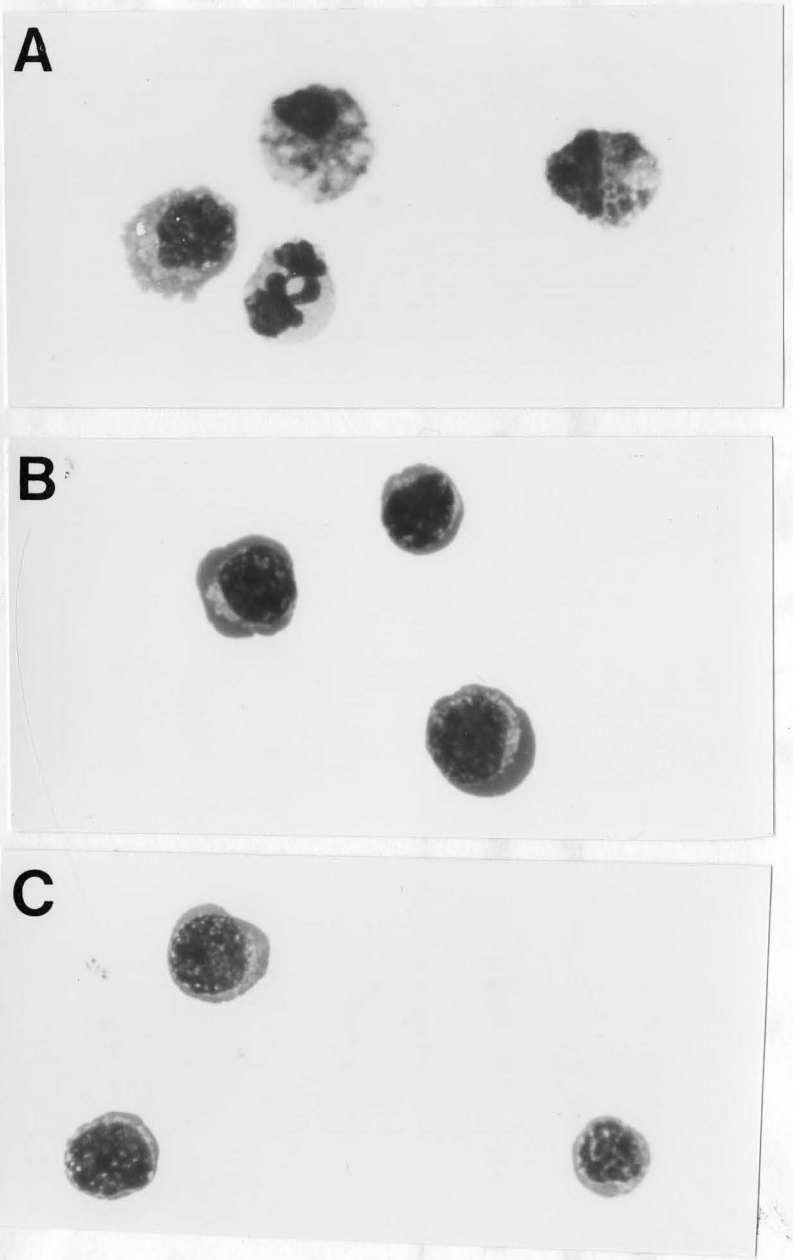
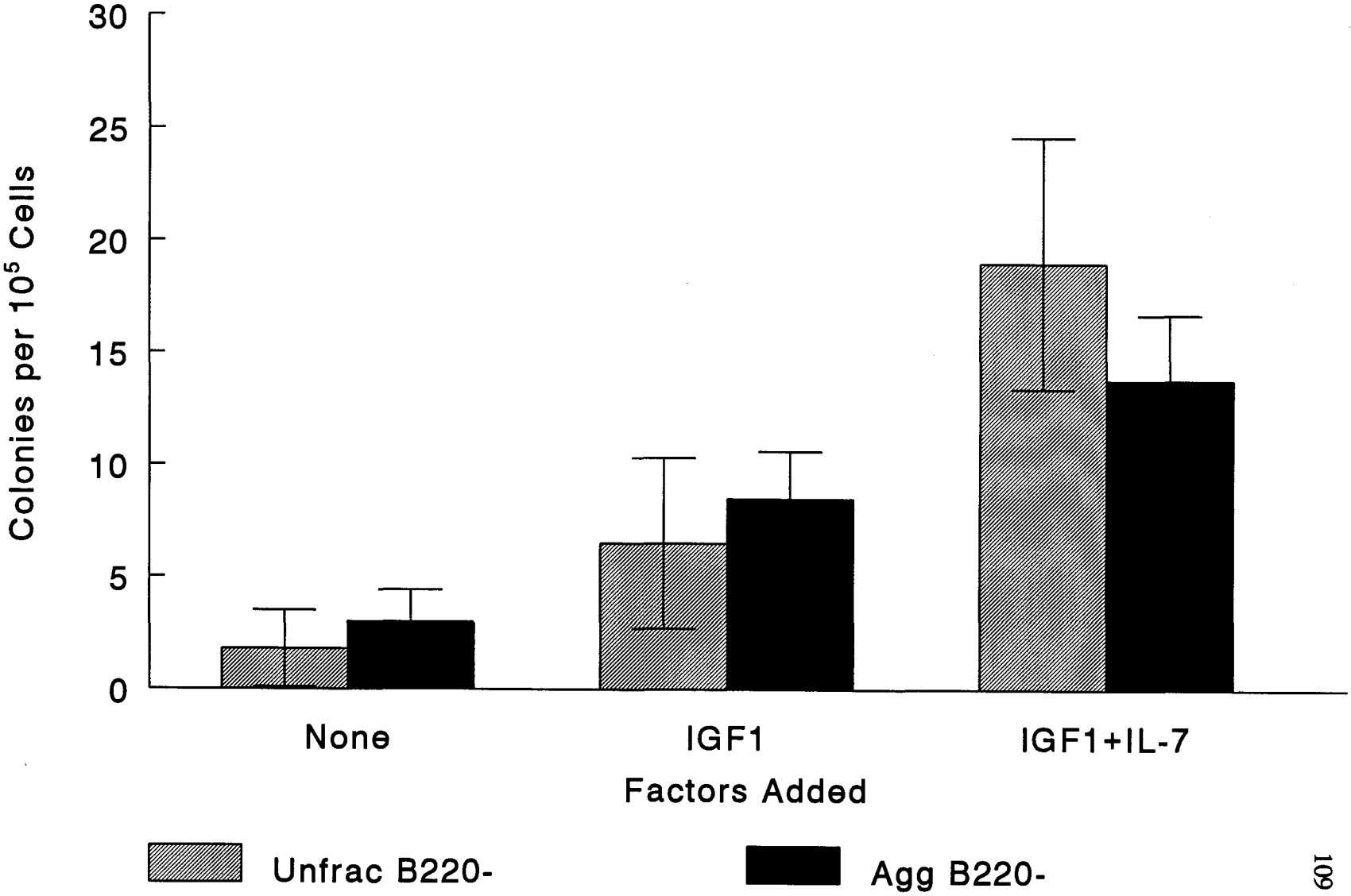

A
B
C

Table 11.--Phenotype of Cells in Colonies

		Percent of Total[a]		
Cells	Condition	Lymphoid[b]	Myeloid[b]	$\mu+$[c]
Unsorted	IL-7	99.0; 98.4[d]	1.0; 1.6	95.3
	IGF-1	12.7; 7.0	76.5; 75.0	N. D.
	IGF-1 + IL-7	95.2; 99.0; 97.2	4.8; 1.0; 2.8	N. D.
B220-	IGF-1 + IL-7	98.1; 95.6; 95.9	1.9; 4.4; 4.1	100[e]

[a] A minimum of 100 cells were scored per slide except where noted. N.D.; Not Done. [b] Assessed by morphology after Jenner/Giemsa stain. [c] Stained for total μ immunofluorescence. [d] Each number represents analysis of pooled colonies from individual experiments. [e] Only 30 cells seen on this slide.

Figure 15. B cell progenitors responsive to IGF-1 + IL-7 in bone marrow fractions. B220- cells were isolated by cell sorting from noncollagenase treated unfractioned marrow or collagenase dispersed aggregates and plated in soft agar culture with growth factors for 6 days. Data shown are the mean $\pm$ S.D. of at least three plates. We have reported that our collagenase treatment does not affect IL-7 responsiveness (3).


30
25
20
15
10
5
0
Colonies per 10^5 Cells
None
IGF1
IGF1+IL-7
Factors Added
Unfrac B220-
Agg B220-

Chapter V

ENRICHMENT OF STROMAL CELLS DIRECTLY FROM BONE MARROW CELL SUSPENSIONS AND ANALYSIS OF THEIR CYTOKINE PRODUCTION

ABSTRACT

Several studies have used *in vitro* derived stromal cell lines to analyze stromal cell functions. However, such cell lines have proven to be heterogeneous by a number of criteria. The primary goal of this dissertation has been to identify and analyze stromal cells directly from bone marrow cell suspensions. In this chapter a gentle centrifugation technique was developed that allows stromal cells to be seen immediately after collagenase digestion of aggregates. Immunomagnetic separation of cells expressing VCAM1 enriches for cells with stromal cell characteristics, enabling histochemical and immunocytochemical studies to be performed. Freshly isolated stromal cells express alkaline phosphatase, VCAM1, α-actin, and about 50% express weak naphthyl esterase activity. These cells also express IL-7 protein and about 50% express KL, but only a few express M-CSF. These results present some contrasts to the stromal cells derived *in vitro*. These studies are the culmination of this dissertation project.

INTRODUCTION

The best avenue to assess biologic activity of any cell type is to study its activity *in vivo*. While this is relatively easy for cell types such as lymphocytes, which can be isolated quickly and in large numbers, stromal cells constitute only a minor fraction of bone marrow cells and are tightly entwined with hemopoietic cells, making their isolation difficult (Chapter II and IV). Stromal cells have been defined in this dissertation by their morphology, alkaline phosphatase activity, and VCAM1 expression. However, stromal cells can only be counted after 48 hours of culture; no attempts to identify fresh stromal cells have been made. Further enrichment of stromal cells is necessary in order to have sufficient quantities for analysis. Since VCAM1 has been found to be constitutively expressed on stromal cells, it seemed logical to use that marker to further enrich stromal cells. Several methods are available to purify cells based on their expression of phenotypic markers: 1) fluorescence activated cell sorting (FACS), 2) panning, and 3) immunomagnetic separation. Each of these techniques has particular advantages and disadvantages so the optimal isolation protocol must be tailored to individual cell types. The objectives of this study are to identify stromal cells directly from cell suspensions of dispersed aggregates and to enrich stromal cells further so that their production of lymphopoietic growth factors can be analyzed.

MATERIALS AND METHODS

Cell Preparation and Isolation of Aggregates. Aggregates were isolated from marrow cell suspensions and dispersed in a cocktail of collagenase and hyaluronidase as described (Chapter II and IV).

Immunomagnetic Separation of VCAM1-Positive Cells. Goat anti-rat IgG (Fc specific) magnetic beads (Advanced Magnetics, Cambridge MA) were incubated for 15 minutes at 4°C with 10μg/ml rat IgG (chromatographically purified, Sigma, St. Louis, MO) or a 1:500 dilution of M/K2 ascites prepared in SCID mice (113,116)(rat anti-mouse VCAM1, American Type Culture Collection, Rockville, MD) then washed three times with RPMI supplemented with 10% heat-inactivated FBS. A bead:cell ratio of 40:1 was chosen by titration experiments based on the bead:cell ration used to isolate endothelial cells (183). Cells were incubated for 20 minutes at 4°C with rat IgG coated beads. Cells that bound rat IgG nonspecifically were then removed using a Biomag Separator (Advanced Magnetics). Negatively selected cells from this step were then incubated for 20 minutes with mAb M/K2 (anti-VCAM1) coated beads. Cells were then subjected to another round of magnetic separation. Immediately after separation and without washing, approximately 10^5 positively selected cells were placed onto Superfrost glass microscope slides (Fisher, Pittsburgh, PA) coated with 10μg of CelTak (Collaborative Biomedical Products, Bedford MA)(99) and incubated at 37°C in a humidified incubator for 1 hour. The slides were then centrifuged at 350rpm in microplate carriers in a Beckman GPR centrifuge with rotor GH3.7 (approximately 30 X g) and subsequently then stained for alkaline phosphatase (Kit 86C, Sigma), acid phosphatase (kit 386-A), naphthyl esterase (kit 91-A), chloroacetate esterase (kit 91-C), or acetyl cholinesterase (184) activity.

Induction of Osteocalcin Expression. The MC-3T3-E1 osteoblastic cell line (185) was kindly provided by Drs. J. Stein and J. Lian, Univ. Mass. Med. Cntr., Worcester, MA.

MC-3T3-E1 cells were cultured in αMEM with 10% FBS. To induce osteocalcin expression E1 cells were cultured in 35mm culture dishes with ascorbic acid and B-glycerol phosphate (Sigma, St. Louis, MO) according to the method of Quarles *et al.* (186) Medium was replaced once a week and cells were used 23 days after being placed under inducing conditions.

Immunocytochemical Staining for Intracellular Cytokine Proteins. Cells on CelTak coated slides were sometimes held in tubes with desiccant overnight before staining. Cells were fixed for 15 minutes in ice cold 95% ethanol with 5% acetic acid. The slides were then washed 3 times in PBS. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol for 30 minutes at room temperature and the slides were again washed 3 times in PBS. At this point the cells were incubated for 1 hour with 50% donkey serum to block nonspecific binding of the primary antibody. Primary antibodies included goat anti-mouse osteocalcin (serum, Biomedical Research Incorporated,), or polyclonal rabbit anti-human IL-7 (affinity purified, cross reacts with mouse, Biosource, Camarillo, CA) using 80μl per slide. The optimal concentration of serum for osteocalcin (1:375 dilution, 4 hour incubation) staining was determined by titration on MC-3T3-E1 cells induced to express osteocalcin. Anti-IL-7 staining was used at a concentration known to stain IL-7 expressing stromal cells from LTBMC-B (0.25-0.5μg, 121). Slides were washed 3 times in PBS and stained with biotinylated donkey anti-goat or donkey anti-rabbit IgG (Fab'2, heavy and light chains, Jackson Immunoresearch, West Grove, PA). After 3 more PBS washes, slides were stained with the Vector ABC Elite kit (1:100, Vector, Burlingame, CA). Horseradish peroxidase enzyme activity was detected

by immersing the slides in 0.45mg/ml diaminobenzidene (DAB, Sigma) for 15 minutes in buffer containing 0.15M NaCl, 0.05M Tris, and 0.07% H_2O_2. Slides were counter stained in hematoxylin, washed, dried, and observed by bright field microscopy.

Antibodies to kit ligand and M-CSF appeared to stain faintly with DAB reaction product on VCAM1 selected cells, so I tried alkaline phosphatase visualization of antibody binding, hoping that the color contrast between positive and negative cells would be easier to judge. Cells to be stained for kit ligand or M-CSF protein were fixed and washed as above. Cells were then incubated with rabbit anti-mouse KL (5μg/ml, overnight incubation, polyclonal, protein A purified, Genzyme, Cambridge, MA) or goat anti-mouse M-CSF (1:200 dilution for 1 hour, serum, kindly provided by Dr. E. R. Stanley, Albert Einstein College of Medicine, Bronx, NY) using 80μl per slide. Both of these antibodies were titered by staining adherent cells from LTBMC-B. After washing and staining with appropriate secondary reagents as above, antibody binding was visualized using an alkaline phosphatase kit (Vector) with blue substrate in the presence of 1μl levamisole per 80μl to block endogenous alkaline phosphatase activity. Slides were counter stained with neutral red and observed by bright field microscopy.

Immunofluorescence Microscopy. Cells on CelTak coated slides were fixed in ice cold 95% ethanol and 5% acetic acid. The slides were then washed 3 times in cold PBS. Following the washes, the cells were stained with 80μl per slide of a 1:300 dilution mAb 1A4 (mouse anti-α-actin, Sigma) for 30 minutes in a humid chamber. Slides were washed again in PBS then stained with 80μl per slide of a 1:30 dilution of FITC conjugated goat anti-mouse IgG (Fab'2, Jackson Immunoresearch) for 30 minutes. The

slides were washed a further 3 times in PBS, mounted in phosphate-buffered glycerol, and observed by epifluorescence microscopy.

RESULTS

Visualizing Stromal Cells from Aggregates. Previous studies have dealt with the enrichment of stromal cells in cell aggregates present in bone marrow suspensions (Chapter II and IV). Therefore, collagenase dispersed aggregates were the starting point in attempting to identify fresh stromal cells. Following gentle centrifugation of cells from collagenase dispersed aggregates onto CelTak coated slides, a rare type of large, alkaline phosphatase positive cell was seen (Figure 16A). Such a cell could not be found on conventional cytocentrifuge preparations. These large cells were usually seen with hemopoietic cells still attached. Their size, oval nucleus, alkaline phosphatase reactivity, and apparently stable attachment to hemopoietic cells were consistent with criteria for stromal cells. Therefore, these cells were tentatively identified as stromal cells. Unfortunately, the low incidence of cells with this morphology precluded preparing them in sufficient numbers for further analysis.

Further Enrichment of Stromal Cells. There are currently no specific markers for stromal cells. VCAM1 has been used as a criterion to identify stromal cells since VCAM1 is expressed on reticular cells in the marrow and in aggregates, on short-term cultured stromal cells, and long-term cultured stromal cells (113,116,121,147, Chapter II). Therefore, further enrichment of stromal cells was attempted based on expression of VCAM1. Initially, FACS was tried because it is a well established method to purify cells and I am fairly familiar with its use. In these experiments collagenase dispersed

aggregate cells were directly sorted into 24 well plates and counted after 48 hours incubation. Fewer stromal cells were found on these plates than were present in the dispersed aggregates before sorting. Turbulence in the sort stream could have destroyed the fragile stromal cells. Therefore, I used increased sort nozzle diameters up to 200μm to slow the sort rate and provide a less turbulent sort stream. These experiments also appeared to destroy stromal cells. Next I tried immunoadsorbent separation on polystyrene plates, commonly called panning, using anti-VCAM coated plates. This technique did deplete the VCAM negative population of stromal cells and, if positively selected cells were left on the plate under LTBMC-B conditions, stromal cells were present after 48 hours incubation. However, removing the positively selected cells from the plate requires scraping, which would likely destroy the stromal cells.

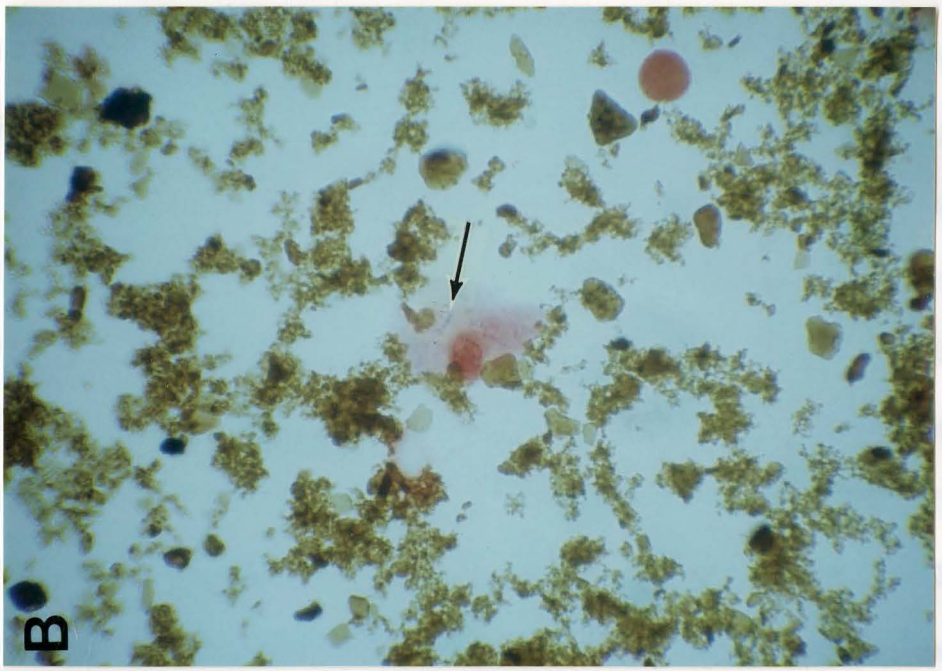
Immunomagnetic separation was used next as a gentler enrichment protocol. Magnetic beads coated with *Ulex europaeus agglutinin* have been used to isolate endothelial cells (183), which may be physically similar to stromal cells. Immunomagnetic separation of VCAM1 positive cells, using 40 beads per cell (183), yielded enrichment of cells with morphology similar to those seen previously from collagenase dispersed aggregates (Figure 16B). In experiments using bead:cell ratios lower than 40:1 these cells did not appear to be as enriched or to segregate exclusively into the VCAM1$^+$ fraction. In 7 experiments, 7.4 $\pm$ 3.0% of collagenase dispersed aggregate cells separated into the VCAM1 positive fraction. The content of stromal cells in the VCAM1$^+$ fraction was verified by morphology and alkaline phosphatase staining. In three separate experiments 23.7 $\pm$ 3.8% of the VCAM1 positive cells had a stromal

cell morphology and almost all of these were alkaline phosphatase positive (Table 12). This leads to an estimate of 1.2 X 10^4 stromal cells in aggregates per femur. This means that stromal cells are 237 times more frequent in VCAM1 positive cells than in collagenase-digested, unselected aggregates and about 700 times more enriched than in unfractioned marrow. However, in must be noted that this is a comparison of the frequency of freshly isolated stromal cells to the frequency of culturable stromal cells in unselected aggregates. Cells with stromal morphology were always surrounded by many magnetic beads, often being partially obscured by them, which suggested that these cells expressed the VCAM1 molecule. Hemopoietic cells were also seen on these slides but were rarely in close contact with magnetic beads, suggesting that they were VCAM1$^-$ contaminants. Some megakaryocytes appeared on these slides as well, always in contact with beads. Identification of megakaryocytes was confirmed by acetyl choline esterase (AChE) staining which clearly distinguished them from the alkaline phosphatase positive cells which were acetyl cholinesterase negative (184).

No cells with stromal cell morphology were seen on identically prepared slides with VCAM1$^-$ cells. The morphology of the alkaline phosphatase positive cells was again quite distinct, being large cells with relatively clear cytoplasm and an oval nucleus. **Based on their morphology, alkaline phosphatase reactivity, and segregation into the VCAM1 positive fraction these cells fulfill all of the criteria used to identify stromal cells throughout this dissertation and will, therefore, be referred to as stromal cells.**

Phenotype of VCAM1 Positive Stromal Cells. Bone marrow contains several cell types which could be the *in vivo* equivalent of cultured stromal cells including endothelial cells,

Figure 16. Alkaline phosphatase positive (blue deposit) cells with stromal morphology. A) Alkaline phosphatase positive cell from collagenase dispersed aggregates on CelTak coated slide. Note hemopoietic cells still attached. B) Alkaline phosphatase positive cell from VCAM1 positive fraction on CelTak slides. Note that alkaline phosphatase activity (small arrow) is much lower after immunomagnetic separation. Also note the magnetic beads surrounding cell. Both photos 630X original magnification.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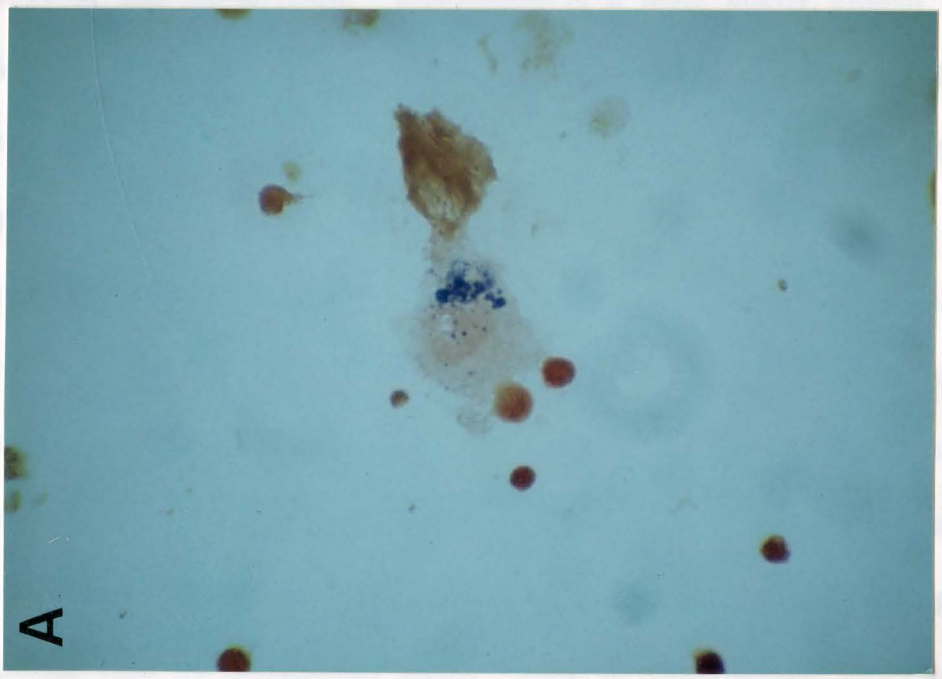

A

osteoblasts, macrophages, reticular cells, and bone lining cells. Therefore, a series of histochemical assays and antibody staining experiments was performed to characterize the putative stromal cells and possibly further define their lineage (Figure 17, 18, and 19, Table 12 and 13). As stated, virtually all cells with stromal cell morphology expressed alkaline phosphatase reactivity, although this activity was weak possibly due to the isolation procedure. Approximately half of the stromal cells exhibited a low level of staining for naphthyl esterase but all were negative for acid phosphatase, chloroacetate esterase, and AChE (Figure 17, Table 13). The level of staining for naphthyl esterase was clearly below that of monocytes from the bone marrow. As well, the regular shape of the nucleus and clear cytoplasm distinguish these cells from monocytes/macrophages (Figure 17 A and B)(187,188). Osteocalcin is a bone matrix protein produced exclusively by osteoblasts, and it has been suggested that marrow stromal cells may be related to osteoblasts (81,103). However, VCAM1 positive stromal cells exhibited undetectable levels of osteocalcin (Figure 18 A, B, and C), suggesting that they are distinct from at least the later stages of osteoblast differentiation. Stromal cells were invariably uninucleate and acid phosphatase negative (Table 13), distinguishing them from osteoclasts (79). Lack of acetyl cholinesterase activity distinguished stromal cells from megakaryocytes (Table 13)(184). In addition, megakaryocytes routinely had a more complex nucleus and cytoplasm than stromal cells, making them easily distinguishable from stromal cells by morphology alone. Lack of chloroacetate esterase activity distinguishes stromal cells from granulocytes (Table 13).

Stromal cells in culture express the α isoform of actin (α-actin)(138,139,140,189).

This actin isoform is normally found in smooth muscle cells and pericytes, cells which line the abluminal wall of small blood vessels outside the reticuloendothelial system (190,191). The stromal cells studied here also possessed α-actin filaments as evidenced by indirect immunofluorescence staining with monoclonal antibody against α-actin, strengthening the comparison between these uncultured VCAM1 positive cells and stromal cells in culture (Figure 19, Table 13). However, the staining of the freshly isolated cells was much less intense than that in stromal cells from LTBMC-B.

Cytokine Protein Production by VCAM1 Positive Stromal Cells. Based on the above results, the $VCAM1^+$ stromal cells appear to be relatively free of potential contaminating cells and are present in sufficient quantity to examine their production of three cytokine proteins, IL-7, kit ligand, and M-CSF, was examined by immunocytochemistry (Figure 20, Table 14). Over 80 percent of the stromal cells expressed IL-7 (Figure 20 A and B), between 50 and 60 percent stained positively for kit ligand (Figure 20 C and D), and less than 7 percent stained with antibodies to M-CSF (Figure 20 E, F, G, and H). No cells staining positively for IL-7 or KL were found among the $VCAM1^-$ cells. Only a small percentage of cells staining for M-CSF appeared among the $VCAM1^-$ cells, these were judged to be monocytes by their morphology. Therefore, stromal cells in the marrow do produce cytokines that help support hemopoiesis, and production of IL-7 and KL appears to be a stromal cell specific function. However, the pattern of cytokines produced by fresh stromal cells can vary considerably from that seen using cultured stromal cell lines (see **DISCUSSION**).

CONCLUSIONS

The results presented here are the first studies to identify and examine marrow stromal cells without intervening culture. This is also the first demonstration of cells from the marrow producing IL-7, kit ligand, and M-CSF. These studies lead to the following conclusions; 1) marrow stromal cells are very fragile and can only be visualized by adopting very gentle centrifugation techniques, 2) marrow stromal cells are rather homogeneous in their expression of alkaline phosphatase and α-actin but heterogeneous in expression of naphthyl esterase, 3) after enrichment by immunomagnetic selection morphologically identifiable stromal cells are distinguishable from osteoblasts, macrophages, hemopoietic cells, megakaryocytes, and most endothelial cells, 4) nearly all stromal cells in young adult mice possess IL-7 protein and about half possess detectable KL protein, 5) in marked contrast to stromal cell lines, few fresh stromal cells possess M-CSF protein.

Table 12.--Cells Expected to Stain with Lineage Markers

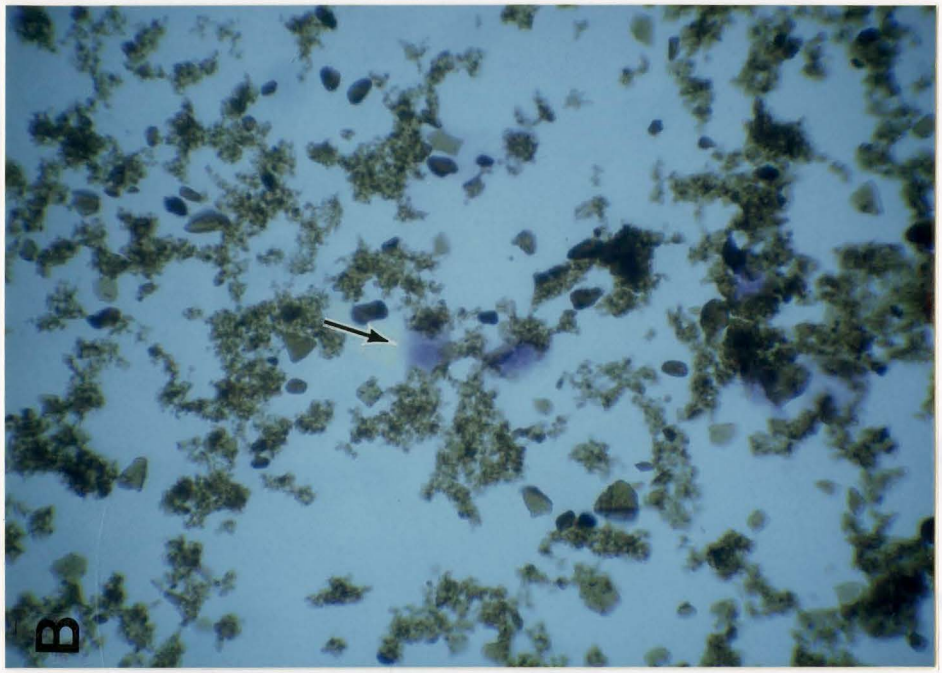
Stain	Positively Staining Cells
Alk. Phos.	Osteoblasts, Fibroblasts, Reticular cells, Granulocytes (weak)
Acid Phos.	Neutrophils, Most Leukocytes
Naph. Estr.	Monocytes
Chloro. Estr.	Granulocytes
AChE.	Megakaryocytes
α-actin	Smooth muscle, pericytes
OC	Osteoblasts

Table 13.--Phenotype of VCAM+ Stromal Cells

Expt.	Percent Positive[a]						
	Alk. Phos.	Acid Phos.	Naph. Estr.	Chloro. Estr.	AchE.	α-actin[b]	OC[c]
1.	86.0	0	57[d]	0	0	94	1
2.	98	0	43	2	0	78	0
3.	96	N.D.	41	N.D.	N.D.	72.7	N.D.

[a] All values are the average of at least two slides, 100 stromal cells were scored per slide. [b] Detected by indirect immunofluorescence, 50 stromal cells scored per slide. [c] Detected by immunocytochemistry, 100 stromal cells scored per slide. [d] Intensity level of naphthyl esterase stain on stromal cells was always lower than that on bone marrow monocytes (see Figure 17).

Figure 17. Naphthyl esterase staining (black dots indicated by small arrows). A) Esterase positive monocyte from VCAM1 negative fraction, 1000X original magnification. B) Esterase negative stromal cell at arrow, original magnification 630X.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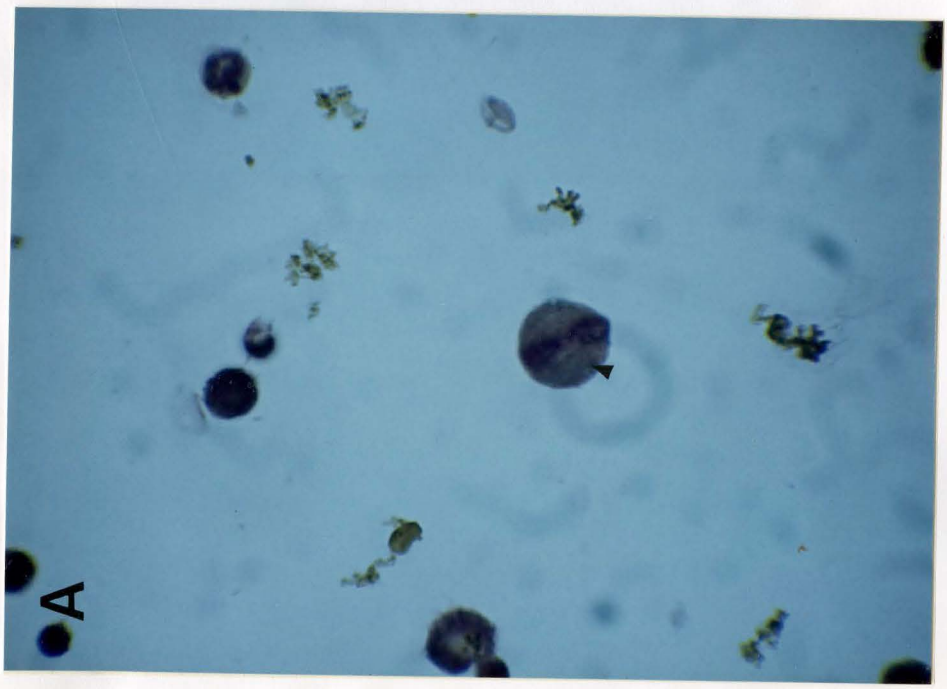
A

Figure 18. Anti-Osteocalcin staining of VCAM1 positive cells. A) MC-3T3-E1 cells induced to express osteocalcin stained with goat anti-osteocalcin antiserum. 630X B) Induced MC-3T3-E1 cells stained with normal goat serum. 630X C) Stromal cell stained with goat anti-osteocalcin antiserum. 1000X

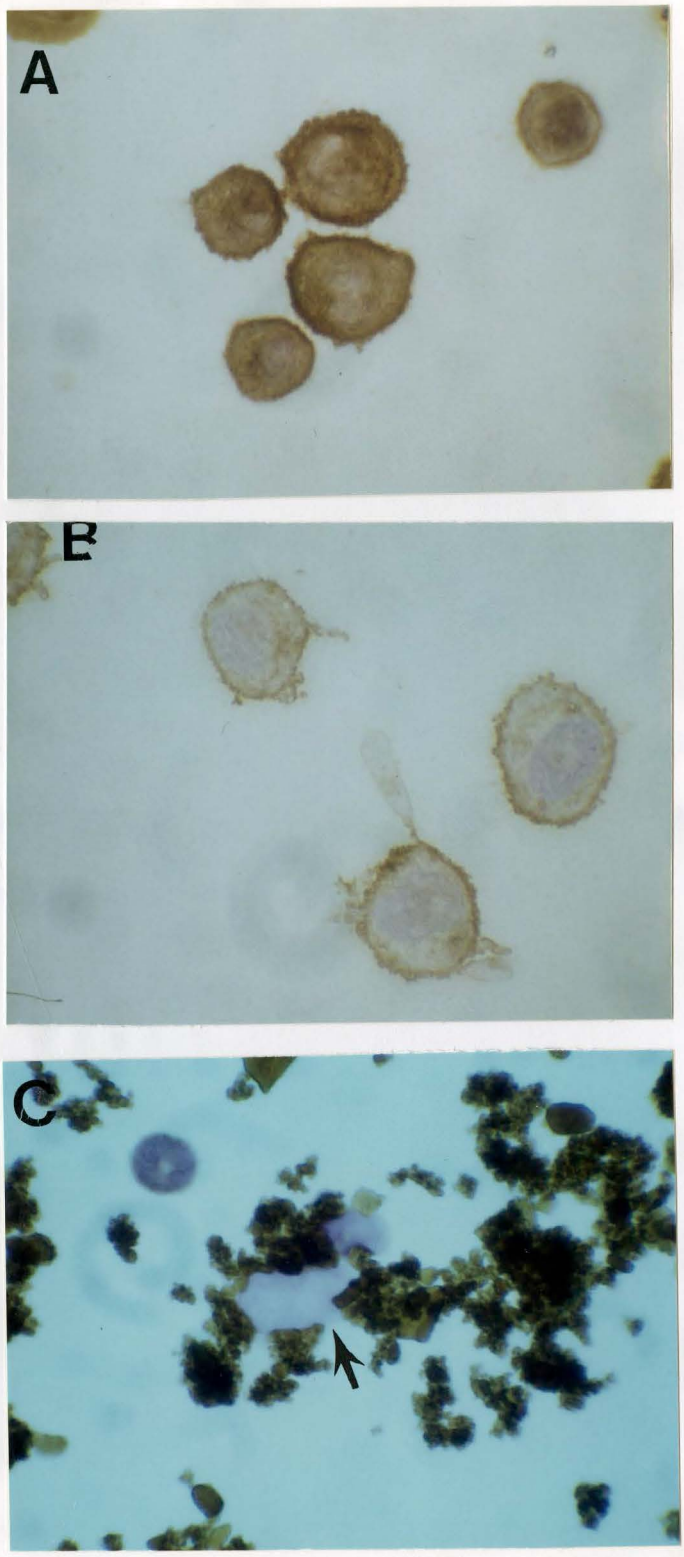
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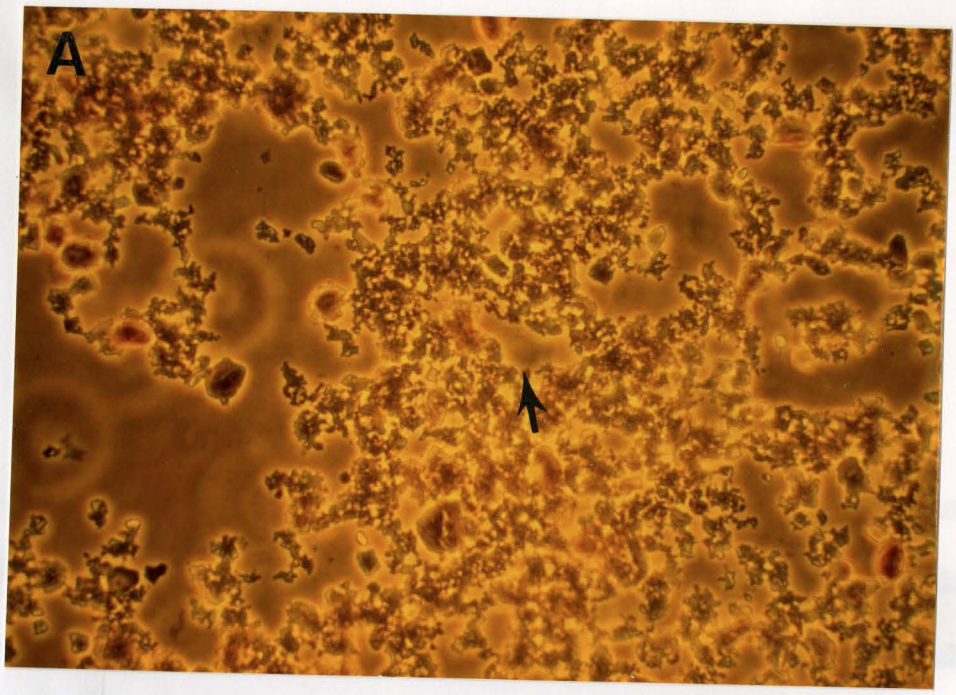
Figure 19. Anti-α-actin staining of VCAM+ stromal cells. A) Phase contrast photomicrograph of VCAM+ stromal cell, note stromal cell nucleus at arrow. 630X B) Fluorescence photomicrograph of same field showing green fluorescence, positive staining for α-actin. 630X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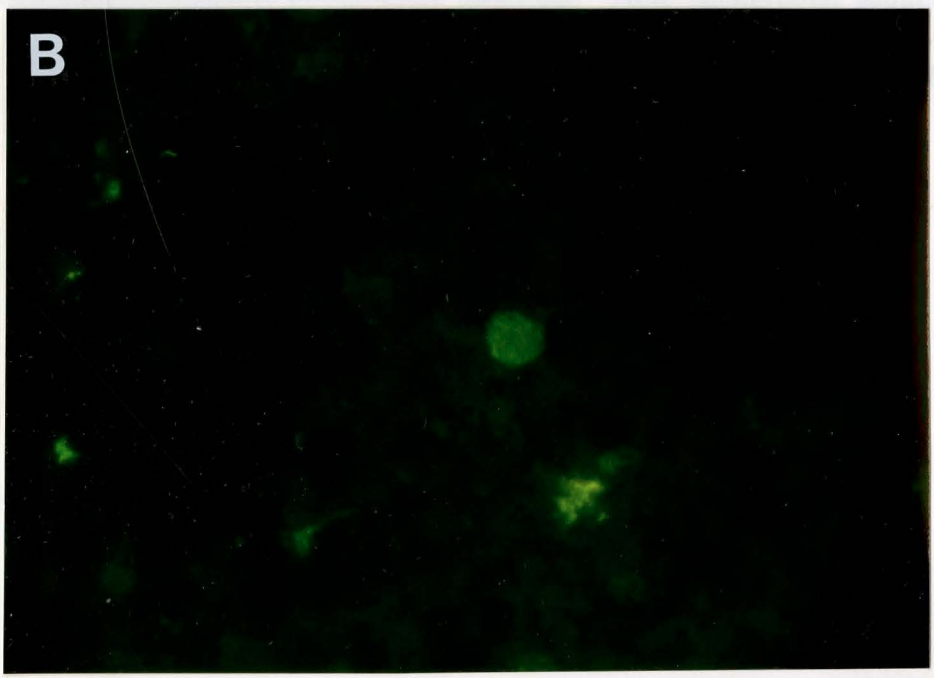
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Figure 20. Anti-IL-7 and Anti-KL staining of VCAM+ stromal cells. A) Stromal cell stained with anti-IL-7, DAB visualization. 1000X B) Stromal cell stained with nonimmune rabbit IgG. 1000X C) Stromal cell stained with nonimmune rabbit IgG, visualised by blue alkaline phosphatase substrate. Note background staining at small arrow. 1000X D) Kit ligand positive stromal cell, note slightly more intense stain at arrow. 1000X

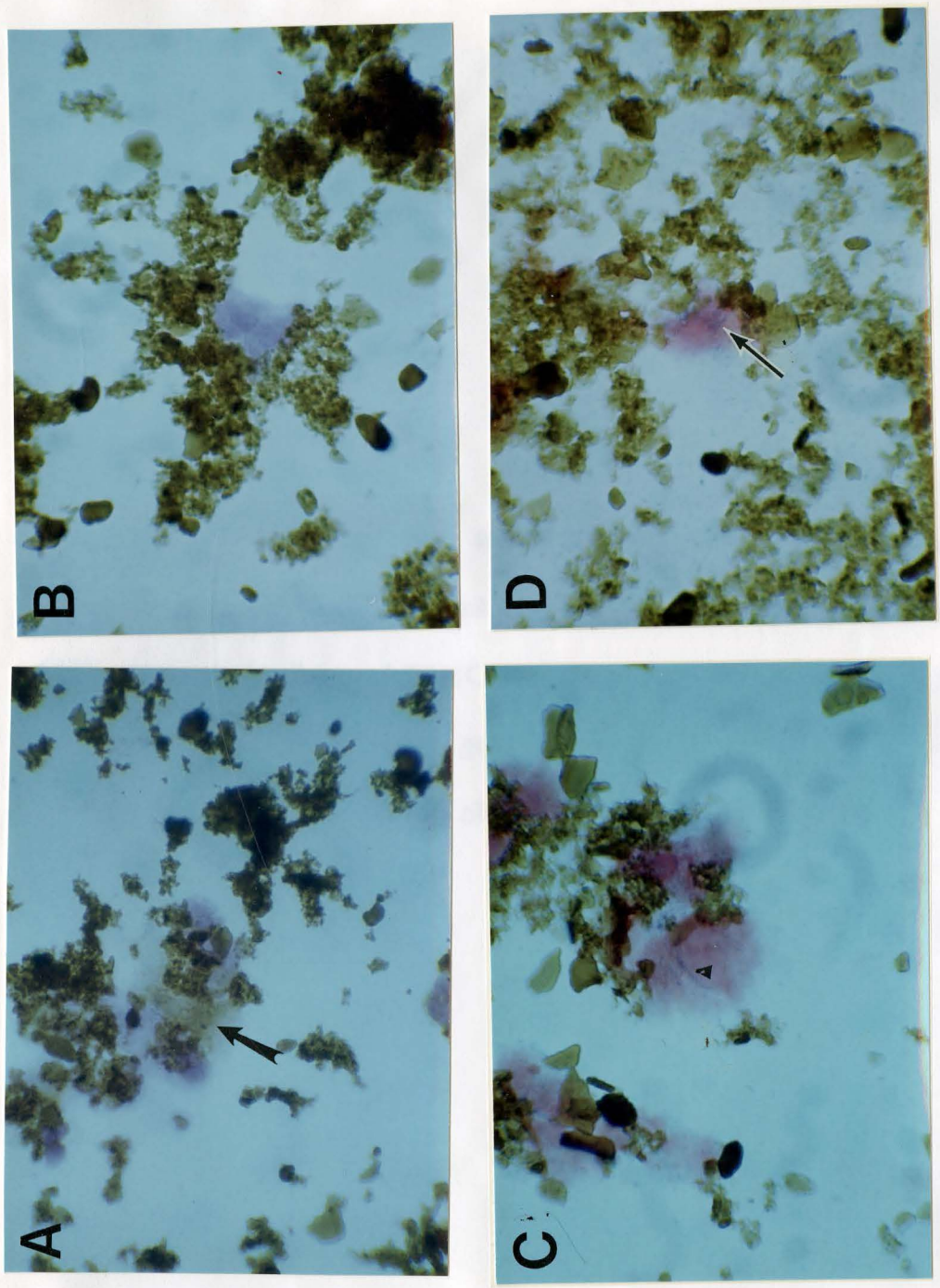
A
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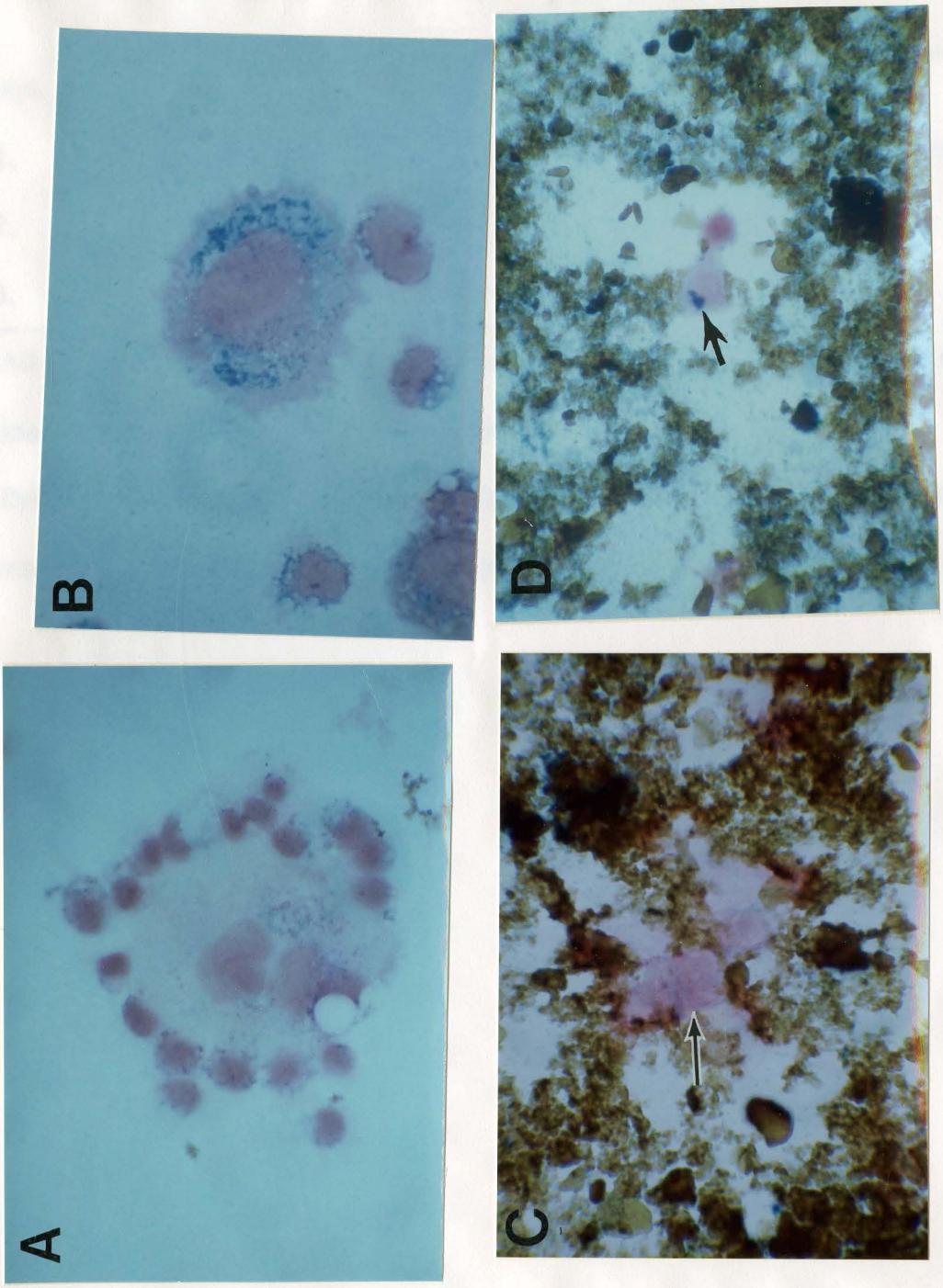
Figure 21. Anti-M-CSF staining of VCAM+ stromal cells. A) Stromal cell from LTBMC-B stained with nonimmune goat serum, alkaline phosphatase blue substrate. 630X B) Stromal cell from LTBMC-B culture stained with goat anti-M-CSF. 630X C) Stromal cell stained with normal goat serum at arrow. 1000X D) Stromal cell with positive staining for M-CSF, note blue deposit at arrow. 1000X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B
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D

Table 14.--Cytokine Protein Production by VCAM+ Stromal Cells

	Percent Positive[a]		
Expt.	IL-7[b]	Kit ligand[c]	M-CSF[c]
1.	83.0 ± 3.0	57.0 ± 6.2	5.0 ± 2.0
2.	84.3 ± 10.7	61.3 ± 4.9	6.3 ± 2.1
3.	84.0 ± 1.0	53.0 ± 1.7	5.3 ± 2.1

[a] All values are the average of three slides ± S.D. 100 stromal cells were scored per slide, i.e. 300 stromal cells per experiment.

[b] Detected by immunocytochemistry with peroxidase/DAB visualization. [c] Detected by immunocytochemistry with alkaline phosphatase visualization.

Chapter VI

DISCUSSION

The exact lineage derivation, location, function, and extent of heterogeneity of the *in vivo* equivalent of cultured marrow stromal cells remain largely unknown. A number of studies have used cultured stromal cell lines and clones in attempts to define the characteristics of stromal cells (3,63,64,122). However, these cell lines and clones are heterogeneous by a number of criteria, making generalizations about stromal cells difficult to draw. Therefore, I began with the goal of studying stromal cells enriched directly from bone marrow cell suspensions. In addition to accomplishing this goal, several interesting facets of stromal cell function and marrow structure have been revealed. The major conclusions of this work are: 1) alkaline phosphatase and VCAM1 are phenotypic markers to define murine bone marrow stromal cells, 2) stromal cells are selectively entwined with hemopoietic cells in the form of cellular aggregates, 3) separation of these cell aggregates results in an enriched population of stromal cells, 4) pre-B cells and TdT+ cells are enriched in the aggregates but clonable B cell precursors are not, 5) aggregates are also enriched in stem cells but not clonable myeloid precursors, 6) adhesion between cells in aggregates is mediated at least in part by VCAM1, 7) separation of VCAM1+ cells from aggregates results in enrichment of cells with all of the hallmarks of stromal cells, 8) the $VCAM1^+$ cells do not possess histochemical reactivity characteristic of other marrow cell lineages, with the exception of weak naphthyl esterase activity, 9) most of these putative stromal cells express the smooth muscle cell (α) isoform of actin, 10) most of these stromal cells express IL-7, about half express KL, and few express M-CSF. Secondary, but still important conclusions of this work are that IL-7 in combination with IGF-1 will generate pre-B

lymphocytes from $B220^-$ marrow, forming a quantitative assay for 'pro-B' lymphocytes, whereas KL in combination with IL-7 will not. These are all important advances in our understanding of bone marrow structure, stromal cell function, and cytokine regulation of B lymphocyte development. In particular, the availability of fresh stromal cells will allow better understanding of the regulation of hemopoiesis *in vivo*.

Identification and Enrichment of Freshly Isolated Stromal Cells

The identification of stromal cells in this dissertation has relied on their morphology, alkaline phosphatase activity, and expression of VCAM1. These markers have been used by other investigators to define both cultured stromal cells and reticular cells *in vivo* (67,113,116,147). In my hands, these markers define a population of large adherent cells with oval nuclei that grow from bone marrow aggregates and adhere and spread in 48 hour cultures of bone marrow. An unavoidable caveat of this work is the lack of a discrete marker for stromal cells. Alkaline phosphatase can be expressed on other cell types and VCAM1 has been found on endothelial cells, synovial cells, and dendritic cells (150). To date, no antibody has been found that specifically identifies bone marrow stromal cells. However, using several criteria in combination should make the identification more precise.

Because I was ultimately interested in isolating normal uncultured stromal cells from bone marrow, I examined the relative frequencies of readily culturable stromal cells from different bone marrow fractions. In murine bone marrow suspensions, stromal cells are found in the highest frequency ($\sim 0.1\%$) in large cellular aggregates as compared to whole marrow or marrow that has been depleted of aggregates. Nevertheless, the

majority of stromal cells are not recovered by our procedures. I estimate that there are about 4000 stromal cells per femur (~0.04% of total marrow) that are identifiable within 48 hours of culture, yet only 800 to 1000 per femur (~25% of total stromal cells) are recoverable from aggregates. Deaggregated marrow, while containing roughly 60% of the nucleated cells of whole marrow, has only about 100 recoverable stromal cells per femur (~2.5% of total stromal cells). Although the number of adherent stromal cells from the central portion of the fetal bovine serum gradient was not determined, initial plating experiments suggested that the smaller aggregates present there contained few culturable stromal cells (Funk, personal observation). That fraction, therefore, is not likely to contain more stromal cells than deaggregated marrow. The question remains, where are approximately 70% of the stromal cells lost during the isolation procedure? It is possible that many stromal cells are destroyed or lost in the collagenase digestion procedure. If this were the case, differences in stromal cell counts would be seen between untreated marrow and unfractioned collagenase treated marrow (Table 3). However, such differences are not apparent in the data. Another possibility is that stromal cells are lost while being placed on or being removed from the FBS gradient. The aggregates themselves appear to be sticky and can be seen adhering to the walls of the pipet while marrow suspensions are being loaded onto the FBS gradient. In addition, removal of aggregates from the gradient with a glass pasteur pipet could lead to shearing of stromal cells in aggregates and would not be reflected in collagenase treated whole marrow counts as these cells did not go through this procedure.

A few previous reports suggested that stromal cells may be present in aggregates

with hemopoietic cells. Lanotte *et al.* (192) directly suggested that stromal cells are present in multicellular aggregates. Whitlock *et al.* (105) commented on the necessity of cell clumps for forming an adherent layer in culture. Crocker and Gordon (144) noted an enrichment of alkaline phosphatase positive fibroblastic cells in clusters separated from marrow suspensions, similar to the findings reported here. Although Crocker and Gordon focused on the enrichment of resident bone marrow macrophages, several key observations were strikingly similar to those in this report. First, both studies show approximately 7 to 8% of total nucleated bone marrow cells are in clusters or aggregates. Second, our data agree that alkaline phosphatase positive fibroblastic cells (presumably stromal cells) are enriched approximately 3 fold in aggregates. Third, both reports document increased ^{3}H TdR incorporation by aggregate cells in comparison with that by whole marrow.

Another comparison to the cell aggregates found in mouse bone marrow is to the hematon described in human marrow (145). However, a number of differences clearly distinguish these elements. First, the hematon is noted for its lack of adherent cells, although hematons contain adipocytes which are common in human marrow and may derive from stromal cells (134), while aggregates are notably enriched for adherent cells. Second, nucleated cells in hematons are in excess of 20% of total nucleated cells in marrow aspirates while aggregates contain, on average, only 7% of cells in femoral marrow. Third, unlike aggregates, hematons are enriched in CFU-GM. Nevertheless, both hematons and aggregates may be representative of similar processes requiring intimate association of stromal elements and developing hemopoietic cells.

IMMUNOMAGNETIC ENRICHMENT OF STROMAL CELLS

Immunomagnetic separation of VCAM1$^+$ cells from dispersed aggregates further enriches cells with stromal morphology to about 23% of all cells present, an enrichment factor of 237 fold over the frequency of culturable stromal cells in aggregates, and nearly 600 fold over the frequency of culturable stromal cells in unfractioned marrow. The previous estimate of the frequency of stromal cells in aggregates was about 700 per femur, based on their ability to attach and spread into morphologically identifiable stromal cells by 48 hours of culture (Chapter II). The data from the VCAM1$^+$ stromal cells leads to an estimate of 1.2 X 10^4 stromal cells per femur in aggregates. If one takes 7 X 10^5 as the average number of cells in aggregates then each stromal cell could interact with about 58 (7 X 10^5/1.2 X 10^4) hemopoietic cells. Another consideration in calculating the number of stromal cells present based on the number of freshly isolated stromal cells is the cell loss that occurs during aggregate isolation and collagenase digestion. If one assumes that 75% of stromal cells are lost in this process as discussed previously, then one calculates approximately 4.8 X 10^4 stromal cells per femur in total bone marrow, or, assuming 1 X 10^7 total cells per femur, stromal cells would constitute 0.48% of the bone marrow cells. Even assuming a 90% loss of stromal cells in this enrichment protocol, stromal cells would make up only 1.2% of marrow cells. These would seem to be fairly realistic estimates of the number of stromal cells within the bone marrow.

ORIGIN OF CULTURED STROMAL CELLS

Comparison of the numbers of freshly isolated stromal cells to culturable stromal

cells in aggregates also suggests that as few as 6% of stromal cells in the marrow ($700/1.2 \times 10^4$) survive in culture for 48 hours. Whether these surviving cells can proliferate to form all of the stromal cells found in LTBMC cultures or if LTBMC stromal cells arise from a mesenchymal precursor is only now amenable to study. Intact aggregates appear essential to the formation of the adherent layer in LTBMC conditions (Chapter II and IV, 105) but, given their rarity, it is unclear if they can account for all of the stromal cells found in mature LTBMC. Other authors have reported the generation of stromal cells from isolated mesenchymal precursors in culture (99,101,198) and this may be the main source of *in vitro* stromal cells. I have been unable to culture the stromal cells isolated by immunomagnetic selection. This could be due to damage to the cells during the isolation process, the beads may interfere with their ability to attach to the culture dish, or these could be mature cells incapable of growing in culture. If methods can be found to culture out these stromal cells their ability to proliferate *in vitro* can be assessed, possibly giving insight into the potential existence of stromal cell precursors. Differentiation of stromal cells from precursors in artificial *in vitro* conditions raises the possibility that the resulting stromal cells possess an equally artificial phenotype, making it unclear if the stromal cells found *in vitro* are equivalent, or even relevant, to the stromal cells *in vivo*. This underscores the importance of studying freshly isolated stromal cells.

EXPRESSION OF VCAM1 BY MARROW CELLS

The close association of the stromal cells described here with the anti-VCAM1 coated beads, as well as their exclusive segregation into the VCAM1 positive fraction,

suggests that they are truly VCAM1 positive. However, since the fresh stromal cells could not be cultured it was not possible to independently confirm their expression of VCAM1. Most of the other cells in the VCAM1 positive fraction were not closely associated with the anti-VCAM1 coated beads, suggesting that about 75% of the selected cells were nonspecific contaminants. Notably, some megakaryocytes were seen attached to the beads. However, some megakaryocytes were also found in the VCAM1 negative fraction. This could mean that these megakaryocytes express VCAM1 or that they passively acquire VCAM1 from other cells during the isolation process. Megakaryocytes in the VCAM1 positive fraction were easily distinguished from stromal cells by their size, cytoplasmic complexity, nuclear morphology, and acetyl choline esterase staining. Such contaminating cells could be present because of the relatively high bead:cell ratio used, making it more likely that cells could nonspecifically associate with beads. However, experiments using lower bead:cell ratios were not as useful in separating stromal cells.

The criteria used to judge stromal cells were rather conservative and have been used by other investigators. This was done so that identification of a cell as stromal could be done with confidence. A limitation with this technique is that the magnetic beads present on the slides could obscure cells. This would introduce some bias into the results since the nuclear morphology of the cell was critical to identification of stromal cells. Such a bias would lead to an underestimation of the number of stromal cells present. Obscuring of cells by the magnetic beads would likely be a random event depending on how the cells settled onto the slides, unless a particular subpopulation of

stromal cells had especially high levels of VCAM1 on the cell surface. If that were the case, that subpopulation, with its potentially unique properties, would not be accounted for in these studies. However, similar numbers of stromal cells were repeatedly found after the immunomagnetic separation protocol, suggesting that this method is reproducible. Electron microscopic analysis of cells enmeshed in the beads would allow any stromal cells present there to be analyzed. The principal cell type that could be confused with stromal cells in these studies was endothelial cells, which comprised about 12% of the cells identified as stromal, as assessed by uptake of acetylated low density lipoprotein. Double staining experiments with endothelial cell markers would allow endothelial cells to be completely eliminated from being scored as stromal cells.

It has been proposed that stromal cell subsets exist that differ in their lympho/hemopoietic support capacity. However, stromal cells in primary culture appear homogeneous by several criteria; including the expression of VCAM1 (113,116,118,119,121), MECA10 (96), 8.28(121), α-actin(138-140,189), collagens I and IV (96), and laminin (96). The fresh stromal cells reported here are also rather homogeneous in their phenotype, being VCAM1$^+$, alkaline phosphatase positive, α-actin positive, and acLDL negative, suggesting that distinct subsets of stromal cells might not be present *in vivo*. This view is also supported by the ability of LTBMC stromal cells to support both lymphopoiesis and myelopoiesis under appropriate conditions (131,135). The only criteria noted here that could define stromal cell subsets are naphthyl esterase activity and expression of KL. Double staining for these markers could determine if they were coincident on the same cells or mutually exclusive. These experiments would be

needed to look for evidence of distinct stromal cell subsets within the marrow.

IN VIVO CORRELATE OF STROMAL CELLS

The most likely *in vivo* correlate of the cells studied here is the reticular cell of the bone marrow. Reticular cells *in vivo* express alkaline phosphatase and VCAM1, as do the stromal cells studied here (66,67,113,147). Reticular cells *in vivo* also possess actin filaments, although the specific isoform of actin has not been determined (65). As yet there is no specific marker for bone marrow reticular cells, so conclusive demonstration that these are reticular cells is not possible. Bone lining cells have only been studied recently and possible phenotypic markers have not been described (73,74), although Westen and Bainton describe alkaline phosphatase activity in the endosteal region of the marrow, possibly including the bone lining cells (67).

The stromal cells described here are not differentiated osteoblasts as they fail to stain with antibodies to osteocalcin, a bone matrix component produced by osteoblasts (80,81). Isolation of osteoblasts requires repeated collagenase digestion of bone slices, so it is unlikely that simply flushing marrow from the bone shaft would dislodge many osteoblasts (80,81,82). These stromal cells can also be distinguished from macrophages by their relatively clear cytoplasm, expression of alkaline phosphatase, and clearly lower levels of naphthyl esterase activity.

The uncultured stromal cells studied here were found to possess α-actin filaments as do stromal cells *in vitro* (138,139,140,189). Smooth muscle cells, while present in the marrow, are extremely rare and are found lining arterial vessels, not among the hemopoietic cells (61). Galmiche *et al.* have recently reported that stromal cells in

culture express other markers of smooth muscle cells (189). However, they report that stromal cells originate from α-actin negative precursors in culture, making it possible that α-actin expression is induced by culture conditions. Nevertheless, expression of α-actin makes it possible that stromal cells *in vivo* are smooth muscle-like, contractile cells with the ability to actively sort hemopoietic cells as adventitial reticular cells are proposed to do (65). Further studies utilizing other markers of smooth muscle cells would be required to determine if stromal cells are smooth muscle in origin. VCAM1 is expressed briefly during development of skeletal muscle (193) but it has not been reported in smooth muscle cells.

Another cell type known to express α-actin is the pericyte, a cell found lining the abluminal side of arterial blood vessels (190,191). One investigation has reported the existence of pericytes in the marrow (19) but other reports have concluded that there are no pericytes in the bone marrow (191,194). It is difficult to propose that the cells isolated here are pericytes since the existence of pericytes in the marrow is controversial.

Some investigators have stated that the large stromal cells in LTBMC-M cultures are endothelial in origin (96). Expression of α-actin also makes it unlikely that these stromal cells are endothelial, although VCAM1 has been reported on marrow sinus endothelium (196). Fresh stromal cells do not stain with the lectin *Ulex europaeus agglutinin I* (UEA)(data not shown), a marker used to identify endothelial cells in other tissues (196-198). However, UEA does not label endothelium in the marrow (199,200) so conclusive discrimination of these stromal cells from endothelial cells will require a more definitive marker of marrow endothelium. An antibody against angiotensin-

converting enzyme has been described as a marker for endothelial cells and may enable discrimination of stromal cells from endothelial cells (201). The coexpression of VCAM1, commonly seen on activated endothelial cells, and α-actin, a smooth muscle characteristic, makes it possible that stromal cells represent a unique lineage with a phenotype intermediate between endothelial cells and smooth muscle. Further investigation with more definitive lineage markers and the discovery of a stromal cell marker would help explore this possibility.

CYTOKINE PROTEIN EXPRESSION BY STROMAL CELLS

Analysis of cytokine protein expression by these stromal cells yielded some interesting comparisons to *in vitro* cultured stromal cells. First, IL-7 appeared to be expressed by nearly all freshly isolated stromal cells. This is surprising in light of the fact that B lineage cells comprise only about 25% of marrow cells and IL-7 responsive B cells are only a small subset of that. It is known that young adult mice, such as those used in my studies, are preferred for setting up LTBMC-B cultures and have active lymphopoiesis (105,173,174). This may explain the widespread expression of IL-7 by stromal cells in young mice. However, the rate of lymphopoiesis slows in older mice (202,203), possibly due to decreased expression of IL-7 by stromal cells in older mice, a question amenable to study by the methods described here. No cells stained for IL-7 protein among the $VCAM1^-$ cells, suggesting that IL-7 staining is a stromal cell specific marker.

Kit ligand has broad hemopoietic effects (38,39,52-58,180-182) but was only found in about half of the stromal cells. The function of KL as only an accessory factor

in B cell formation, as well as its potent effects as a synergistic factor in combination with other hemopoietic cytokines, may mean that widespread expression of KL is not essential. Rather, small, focal amounts may be more effective. Nevertheless, this is the first report of KL expression by stromal cells in the marrow; previous studies have found expression of kit ligand on cultured stromal cells and extrapolated its *in vivo* function from that data (38,39,52-60,206). Similarly to IL-7, no cells stained for KL protein in the VCAM1$^-$ fraction.

A particularly revealing comparison between these uncultured stromal cells and cultured stromal cells is the expression of M-CSF. M-CSF is expressed constitutively by primary cultured stromal cells and by all stromal cell lines (3,63,64,120,121,122,129). Based on this data, it has been suggested that M-CSF production by stromal cells is important *in vivo*, possibly to maintain resident marrow macrophages (122). Very few freshly isolated stromal cells possess detectable M-CSF protein, suggesting that M-CSF expression by cultured stromal cells may be induced by culture conditions. As well, endothelial cells are known to produce M-CSF. A small contamination of the stromal cells with endothelial cells could explain the M-CSF staining, meaning that possibly no stromal cells possessed M-CSF protein. About 2% of the VCAM1$^-$ cells stained positively for M-CSF protein. These positive cells were determined to be monocytes by morphology.

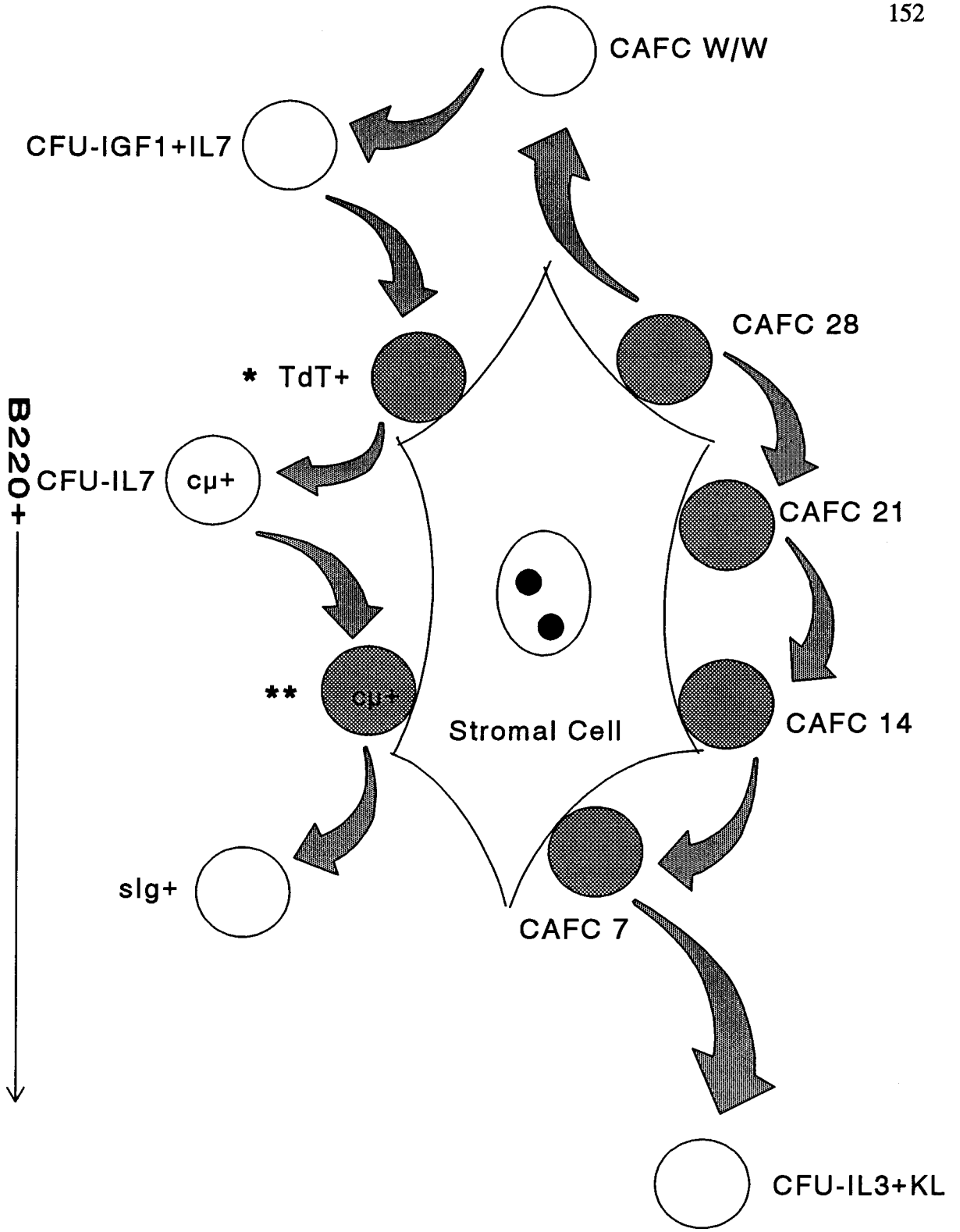
These cytokine studies establish a baseline expression of three hemopoietic cytokines by marrow stromal cells in young mice. Some evidence suggests that the rates of lymphopoiesis and myelopoiesis are inversely related. It may, therefore, be useful to

study changes in cytokine production in times of hemopoietic recovery as in irradiation or after treatment with cytotoxic drugs such as 5-fluorouracil (44,205).

Bone marrow aggregates represent normal associations of stromal cells with specific stages of hemo/lymphopoiesis.

These studies focused on the frequency of cells in aggregates because an increase in frequency means that cells were not randomly distributed throughout the marrow, but selectively retained in one fraction or the other. Since aggregates contain such a small proportion of total marrow cells they would be expected to contain only a minor fraction of the absolute number of any given cell type. Enrichment of cells of a particular differentiation stage within the aggregates implies a necessity for contact or close association with stromal cell processes, which interdigitate through the aggregates. Other cell types examined were found to be present in equal frequency in aggregates and in unfractioned marrow, meaning that the distribution of these cells is random within the marrow. Alternatively, the dissection of the cell populations may not be discrete enough and subpopulations of certain stages may be sequestered in aggregates. Additional markers for B lymphocyte development would allow for better scrutiny of cells requiring stromal cell contact. Potentially, the cells in aggregates could, in and of themselves, define subsets of marrow populations. For example, differences in proliferative activity, marrow repopulating ability, or mobility of stem cells may exist. It must also be noted that previous analysis of bone marrow, with a few exceptions (105,144,145,192), has neglected the existence of cell aggregates, potentially biasing results concerning stem cell populations.

Figure 22. Scheme of hemopoietic cell-stromal cell contacts occurring in marrow aggregates. Cells filled with hatched patterns are known to be enriched in aggregates (3, this report). Open circles represent cells not enriched in aggregates. Arrows denote differentiation pathways for hemopoietic cells. Cells thought be rearranging IgH (*)(31) and IgL (**)(36) are indicated. CAFC 28, presumably the most primitive cell type shown, give rise to more mature CAFC and eventually to colony forming units responsive to IL-3 and KL. Cells forming cobblestones in LTBMC-B conditions and B lineage colonies in response to IGF-1 and IL-7 do not express B220(CD45RA) and are therefore the most primitive B cell precursors shown. Although only one stromal cell type is shown it is possible that different types of stromal cells exist *in vivo*.


CAFC W/W
CFU-IGF1+IL7
CAFC 28
* TdT+
B220+
CFU-IL7
cμ+
CAFC 21
**
cμ+
Stromal Cell
CAFC 14
sIg+
CAFC 7
CFU-IL3+KL

While it has not been definitively tested here, I believe that these aggregates are present naturally within the marrow and may represent native intercellular associations. It is unlikely that aggregates represent collapsed tissue sinuses in the marrow. Intact marrow sinuses are filled with IgM^+ B cells awaiting egress from the marrow (68) and, if collapsed in our marrow preparations, would be expected to be enriched in IgM^+ cells. Our aggregates, however, are actually depleted of $s\mu^+$ B cells, arguing against a sinusoidal origin of the aggregates. Moreover, the aggregates are resistant to vigorous pipeting or vortexing, procedures that would be expected to disrupt non-specific interactions. A tight association of the cells is implied by the comparatively harsh collagenase conditions used, which are the minimum required to completely disperse the clusters. The numbers of cells recovered in aggregates is remarkably constant from experiment to experiment suggesting that they are a reproducibly isolated component of the normal marrow. In addition, the reticular staining patterns reported for mAb M/K2 and alkaline phosphatase in whole marrow (67,113) is reproduced in isolated aggregates (Fig. 1) supporting the belief that aggregates mirror the native architecture of regions within the marrow. As well, disruption of the aggregates *in vivo* by injection of anti-VCAM1 antibody strongly suggests that the aggregates are present naturally.

VCAM1 AS AN ADHESION MOLECULE IN THE MARROW

These results confirm a role for VCAM1 in stromal-hemopoietic cell adhesion *in vivo*. The failure of these experiments to disrupt all aggregates could be due to several effects: 1) the amount of antibody injected was not optimal; 2) the time after injection was not optimal; 3) not all stromal cells use VCAM1 at any given time; 4) not all

aggregates contain stromal cells (Chapter II). Moreover, the closure of sinusoids during 'lymphocyte loading' could decrease blood flow to localized regions of the marrow, thus decreasing the local concentration of antibody and allowing some aggregates to escape disruption over the short time span (68). The time after injection was chosen based on previous reports showing that bone marrow cells could be efficiently labelled with antibody as rapidly as 15 minutes after *i.v.* injection (147). Although I have not determined how much antibody remains in circulation when the marrow is harvested, I consider it unlikely that such residual antibody leads to the aggregate disruption seen here. The marrow is immediately flushed from the femur, in this process the original marrow volume is diluted 20 to 30 fold. It is unlikely that such dilute antibody could mediate aggregate disruption, particularly in light of attempts to see aggregate disruption in vitro, which used 20μg/ml of antibody to see an effect. The aggregates that survived anti-VCAM1 antibody treatment contained stromal cells in frequency identical to that in untreated control animals (data not shown). These remaining stromal cells could use adhesion mechanisms other than VCAM1. However, since the absolute number of stromal cells in aggregates was reduced it is likely that some stromal cells were entirely liberated from aggregates, implying that at any one time some fraction of stromal cells may solely use VCAM1 as an adhesion mechanism.

Only anti-VCAM1 antibodies were tested in these studies because VCAM1 seems to be expressed on stromal cells and very little else in the marrow (113). VLA4, a ligand for VCAM1, is expressed on a variety of cell types (116). For example, many hemopoietic cells, including stem cells, express VLA4, a ligand for VCAM1, but *in vitro*

these cells bind fibronectin as well as VCAM1$^+$ stromal cells (118,119,206). Therefore, using anti-VLA4, it would not be possible to attribute the disruption to either ligand. Also, the presence of VLA4 on a high percentage of bone marrow cells would probably necessitate a high dose of antibody to see effects. Similar arguments also apply to another stromal-lymphopoietic cell adhesion molecule, CD44(Pgp1), which is also expressed on >90% of bone marrow cells and can bind hyaluronate in the extracellular matrix (114,117). Nevertheless, it is clear that many aggregates are held together by mechanisms involving VCAM1 and that this is an important adhesive interaction used by stromal cells both *in vivo* as well as *in vitro*.

AGGREGATES APPEAR TO ORIGINATE IN THE SUBENDOSTEAL MARROW.

A gradient of differentiated B lineage precursors exists in the marrow; the least mature stages predominate in the peripheral, endosteal areas and more mature stages reside in the central marrow, distal to the bone (31,44,45,207). Aggregates bear some features that suggest that they originate primarily in the subendosteum. For example, TdT$^+$ cells are reported to constitute 4% of endosteal cells and to diminish to 0.7% as one progresses toward the central marrow (45). These percentages are parallelled in our data on aggregates and deaggregated marrow, respectively. The proliferative activity of marrow, as assessed by ^{3}H TdR incorporation and autoradiography, is also greater peripherally than centrally (208). Again, this coincides with our results showing greater ^{3}H TdR incorporation by aggregate cells than deaggregated marrow. The apparent inconsistency between the marked increase in ^{3}H TdR uptake and the modest increase in percentage of cells in S + G_2M in aggregates might be resolved by interpreting these

results in the light of reports that endosteal cells, i.e. more primitive progenitors typified by TdT^+ cells, are proceeding through the cell cycle very quickly (173). Over the 18 hour pulse period used in these experiments such immature progenitors would, therefore, go through more mitoses than more differentiated, less mitotically active cells present in deaggregated marrow, thus incorporating more radioactive label. Therefore, it is entirely plausible that aggregate cells, while containing only slightly more cells in S + G_2M, are actually capable of incorporating much more radioactive label than deaggregated marrow cells.

STROMAL CELL-HEMOPOIETIC CELL ASSOCIATIONS

Since aggregates appear to represent normal associations in the marrow, I attempted to dissect the stages of hemopoiesis that are enriched in aggregates. Such an analysis should give clues to the stages of hemopoiesis that normally interact with stromal cells in the marrow. The content of precursor cells within different hemopoietic lineages in aggregates was analyzed because an increase in frequency means that cells were not randomly distributed throughout the marrow, but were selectively retained in one fraction or the other. The results of these studies are schematized in Figure 22, highlighting the stages of hemo/lymphopoiesis that, by being enriched in the aggregates, may require association with stromal cell processes. The most primitive CAFC (day 28) are proposed to give rise to progressively more mature CAFC (days 21,14,7) and eventually to CFU-S (25), which can proliferate in response to IL-3 and KL (180). In this scheme it is only the CAFC that selectively interact with stromal cells.

The interactions of developing B cells appear to be more complex. The earliest

detectable B cell precursors, those forming cobblestones after 14 days of culture under lymphoid permissive conditions (CAFC-W/W) (177,209) and those responding to IGF-1 and IL-7 (40), do not appear to require stromal cell association. Cells expressing TdT, and presumably undergoing IgH chain rearrangement (31), are enriched within aggregates (Chapter II)). Cells expressing Igμ chain in the cytoplasm (cμ+) are enriched in aggregates but IL-7 responsive cells, a subset of cμ+ cells (34), are not enriched (Chapter II). Therefore we predict that a cμ+ cell that has proceeded past IL-7 responsiveness and may be undergoing IgL chain rearrangement requires stromal cell association, as proposed by Melchers and colleagues (36). Although we show only one stromal cell in this scheme it is entirely possible that multiple stromal cell types are present within the aggregates and the associations shown may be specific to particular stromal cell types.

DEVELOPMENT OF A QUANTITATIVE 'pro-B' CELL ASSAY

An important aspect of this work was the development of a quantitative assay for very primitive B cell precursors. This work has shed new light on the effects of KL and IGF-1 on B lymphocyte development. KL may not be obligate for B cell development in homeostatic adult animals. For the most part, the synergistic activity of KL and IL-7 *in vitro* seems limited to increasing colony size rather than the number of colonies. This was observed in 5 of 6 experiments. The increased number of colonies from $B220^+$ cells in experiment 2 still implies a role for KL in the B lineage. The presence of c-kit on a small fraction of $B220^+$ marrow cells (162) as well as on a number of $B220^+$ cell clones representing the earliest stages of B cell development (37,210) also implies that KL may

act on the B lineage, but only as a proliferative costimulus with IL-7 after the onset of B220 expression. Treatment of normal animals with an antibody that inhibits c-kit function leads to a massive increase in the percentage of B lineage cells in the marrow (162), again arguing that KL is not essential to B lymphopoiesis. However, the status of B cell development and function in KL or c-kit deficient *Sl/Sl*^{d} and *W/W*^{v} animals remains controversial (211-213). B cell precursor numbers are normal in the adult mice but depressed in fetal liver, and marrow cells from *W/W*^{v} animals are inefficient in regenerating B cells in irradiated normal recipients (175,176).

A colony assay for B220(CD45RA)$^{-}$ B cell precursors in the bone marrow, based on the work of Landreth *et al.*(40), was devised. This approach made it possible for us to detect an earlier, possibly uncommitted, B cell precursor. Previously, the earliest B cell precursor examined was the TdT^{+} stage, which is enriched within the aggregates (Chapter II). Given this, it is puzzling that B220(CD45RA)$^{-}$ B cell precursors responsive to IGF-1 and IL-7 are not enriched in aggregates. These cells are reported to become μ^{+} within 48 hours of culture with IGF-1 and IL-7 (40) and presumably are predecessors of TdT^{+} cells. It is unknown if these B cell precursors express TdT and proceed through normal IgH chain rearrangement and N region addition *in vitro*. There could be a stromal cell specific interaction required to express TdT, explaining why TdT^{+} cells are sequestered in aggregates while earlier B cell precursors are not. Alternatively, the interaction between very early B lymphoid precursors and stromal cells could be more labile. A unique molecule, recognized by mAb KMI6, is found solely in areas of lymphoid precursor interaction with stromal cell processes in the marrow (147). The

tightness of this adhesion is unknown.

Muller-Sieburg *et al.* (177,209) has reported a limiting dilution system to detect lymphoid restricted precursors, similar to the CAFC technique, using a single readout time of 14 days. The relationship of this precursor to the cell responding to IGF-1 + IL-7 is unclear, although neither cell expresses the B lineage antigen B220(CD45RA). Using this lymphoid cobblestone assay (CAFC-W/W), I was again unable to find a difference in the frequency of this cell between the aggregates and unfractioned marrow (data not shown). This strengthens the conclusion that many of the earliest B cell progenitors are not sequestered in aggregates, but that specific stages of B cell genesis are found, presumably requiring stromal cell signals at restricted points of development.

DIFFERENTIAL REQUIREMENTS FOR ADHESION TO STROMAL CELLS

These results may reflect differential requirements for stromal cell contact by myeloid and lymphoid progenitors. In this study early stem cells, culturable when in contact with stromal cells, were found to be enriched in aggregates. Later stage cells, capable of proliferation in soft agar culture without stromal cell contact, were not enriched in aggregates, not were they specifically excluded. This differential sequestering may reflect less stringent binding of the nonenriched precursors by stromal cells, making them more easily sheared off of the stromal cells. The diffusability of growth factors and their activity when bound to extracellular matrix components could make it unnecessary for some cells to be confined to stromal cells in aggregates (214,215). Some cells might be excluded from the aggregates but are able to adhere

nonspecifically to aggregates or they may settle into the aggregate fraction in stromal cell-less clumps, increasing their apparent frequency. They might also have an intrinsically high density, causing them to settle into the aggregate fraction. The simplest explanation, and probably the most likely, is that these cells are randomly distributed throughout the marrow and partition equally into both aggregates and deaggregated fractions.

Aggregates do not exclusively retain all cells of any given differentiation stage. Cells not retained by the aggregates could be striped off of stromal cells during the isolation procedure or the stromal cell might be destroyed as the marrow is flushed from the bone, liberating the attached cells. Alternatively, aggregates might define unique subsets of marrow cells differing in activation state, marrow repopulating ability, or proliferative potential. Additional markers could help to further dissect the cells contained in the aggregates.

FUTURE DIRECTIONS

The discovery that stromal cells are selectively entwined in cellular aggregates that are also enriched in selected stages of hemopoietic development has allowed for a novel means to study stromal cell interactions and marrow structure *in situ*. In addition to summarizing a good deal of data on the hemopoietic steps shown to be enriched in aggregates, the scheme presented in Figure 22 is a framework for future experiments. For instance, experimental manipulations are available to deplete the marrow of hemopoietic cells. Injection of retrovirally marked stem cells after such treatment would allow them to be followed through development as they enter and leave the aggregates.

This would allow a more discriminating analysis of stromal cell dependent steps as well as an avenue to study cell migration within the marrow. Blaszek *et al.* reported that the Hematon fraction was reduced in patients with myelodysplastic syndromes or myeloid leukemias (145). Similarly, the fraction of marrow cells present in aggregates might be used as an indicator of marrow health.

Changes in cytokine production during marrow regeneration could be studied using the methods described in the dissertation, allow the relative importance of cytokines to be assessed. For instance, treatment of animals with 5-fluorouracil (5-FU) rapidly depletes hemopoietic cells from the marrow (205). The marrow then repopulates each lineage of hemopoietic cells in a characteristic order. Stromal cells could be isolated and their cytokine production analyzed to see if the pattern of cytokine production changes as each of the blood cell lineages repopulates. In addition, it has recently been reported that lymphopoiesis decreases as mice age (202,203). The mechanism of the decrease is unknown but it could be due to a decreased production of lymphopoietic cytokines by stromal cells. Study of fresh stromal cells as reported here would be a way to test this hypothesis.

This dissertation has yielded new insights into a number of areas of hemopoiesis. First, the study of the stromal cell enriched bone marrow aggregates has provided a new model to study marrow structure and stromal cell-hemopoietic cell interaction *in vivo*. Second, the identification and enrichment of stromal cells directly from the marrow allows the study of stromal cells without the caveats of cell changes during culture. Third, these results demonstrate that stromal cells *in vivo* produce hemopoietic cytokines,

similar to those found in LTBMC-B cultures. Fourth, the development of a quantitative assay for $B220^-$ B cell progenitors will allow other investigators to study very early stages of B lymphopoiesis. This dissertation not only provides new data about marrow structure and stromal cells, it provides many new avenues for future research into the control of hemopoiesis.

REFERENCES

1. Gordon M. Y., and A. J. Barrett. 1985. Bone Marrow Disorders: The Biological Basis of Clinical Problems. Blackwell Scientific Publications Oxford, UK

2. Bloom W., and D. W. Fawcett. 1986. Textbook of Histology, W. B. Saunders Company

3. Kincade, P. W., G. Lee, C. E. Pietrangeli, S. I. Hayashi, And J. M. Gimble. 1989. Cells and Molecules that Regulate B Lymphopoiesis in Bone Marrow. Ann. Rev. Immunol. 7:111.

4. Tavassoli, M. 1989. Fatty Involution of the Marrow and the Role of Adipose Tissue in Hemopoiesis. in: Handbook of the Hemopoietic Microenvironment. M. Tavassoli ed. Humana Press

5. Carnot, P. 1906. Sur le Mechanisme d'Hyperglobulie Provoquee par le Serum d'Animuax en Renovation Sanguine. CR Acad. Sci. 111:344

6. Miyake, T., CK-H. Kung, and E. Goldwasser. 1977. Purification of Human Erythropoietin. J. Biol. Chem. 252:5558

7. Goldwasser, E. 1989. Erythropoietin: Molecular and Cellular Biology. in: Hematopoietic Growth Factors and Transfusion Medicine. J. Spivak, W. Drohan, and D. Dooley (Eds.) Wiley-Liss

8. Lichtman, M. A., C. H. Packman, and L. S. Constine. 1989. Molecular and Cellular

Traffic Across the Marrow Sinuses. in: Handbook of the Hemopoietic Microenvironment. M. Tavassoli ed. Humana Press

9. Seelentag, W. K., J. J. Mermod, R. Montesano, and P. Vassalli. 1987. Additive Effects of Interleukin-1 and Tumor Necrosis Factor-α on the Accumulation of the Three Granulocyte and Macrophage Colony-Stimulating Factor mRNAs in Human Endothelial Cells. EMBO J. 6:2261

10. Zsebo, K. M., V. N. Yuschenkoff, S. Schiffer, D. Chang, E. McCall, C. A. Dinarello, M. A. Brown, B. Altrock, and G. C. Bagby. 1988. Vascular Endothelial Cells and Granulopoiesis: Interleukin-1 Stimulates release of G-CSF and GM-CSF. Blood 71:99

11. Zucali, J. R., H. E. Broxmeyer, C. A. Dinarello, M. A. Gross, and R. S. Weiner. 1987. Regulation of Early Human Hematopoietic (BFU-E and CFU-GEMM) Progenitor Cells *in vitro* by Interleukin-1 induced Fibroblast-Conditioned Medium. Blood 69:33

12. Broudy, V. C., K. S. Zuckerman, S. Jetmalani, J. H. Fitchen, and G. C. Bagby. 1986. Monocytes Stimulate Fibroblastoid Bone Marrow Stromal Cells to Produce Multilineage Hematopoietic Growth Factors. Blood 68:530

13. Zucali, J. R., C. A. Dinarello, D. J. Oblon, M. A. Gross, L. Anderson, and R. S. Weiner. 1986. Interleukin-1 Stimulates Fibroblasts to Produce Granulocyte-Macrophage Colony-Stimulating Activity and Prostaglandin-E2. J. Clin. Invest. 77:1857

14. Zuckerman, K. S., G. C. Bagby, E. McCall, B. Sparks, J. Wells, V. Patel, and D. Goodrum. 1985. A Monokine Stimulates Production of Human Erythroid Burst-Promoting Activity by Endothelial Cells *in vitro*. J. Clin. Invest. 75:722

15. Rennick, D., G. Yang, L. Gammell, and F. Lee. 1987. Control of Hemopoiesis by a Bone Marrow Stromal Cell Clone: Lipopolysaccharide- and Interleukin-1-Inducible Production of Colony-Stimulating Factors. Blood 69:682

16. Miller, M. L., and R. S. McCuskey. 1973. Innervation of Bone Marrow in the Rabbit. Scand. J. Heamatol. 10:17

17. Calvo, W. 1968. The Innervation of the Bone Marrow in Laboratory Animals. Am. J. Anat. 123:315

18. Calvo, W., and J. Forteza-Vila. 1970. Schwann Cells of the Bone Marrow. Blood 36:180

19. Yamazaki, K., and T. D. Allen. 1990. Ultrastructural Morphometric Study of Efferent Nerve Terminals on Murine Bone Marrow Stromal Cells, and the Recognition of a Novel Anatomical Unit: the "Neuro-Reticular Complex". Am. J. Anat. 187:261

20. DePace, D. M., and R. H. Weber. 1975. Electrostimulation and Morphologic Study of the Nerves to the Bone Marrow of the Albino Rat. Acta. Anat. 93:1

21. Weber, R. H., R. DeFlice, R. J. Ferguson, and J. P. Powell. 1970. Bone Marrow Response to Stimulation of the Sympathetic Trunks in Rats. Acta. Anat. 77:92

22. Uchida, N., W. H. Fleming, E. J. Alpern, and I.L. Weissman. 1993. Heterogeneity of Hematopoietic Stem Cells. Curr. Opin. Immunol. 5:177

23. Keller, G. 1992. Hematopoietic Stem Cells. Curr. Opin. Immunol. 4:133

24. Till, J. E.,and E. A. McColluch 1961. A Direct Measurement of the Radiation Sensitivity of Normal Mouse Bone Marrow Cells. Radiat. Res. 14:213

25. Ploemacher, R. E., J. P. van der Sluijs, J. S. A. Voerman, and N. H. C. Brons.

1989. An *in vitro* Limiting Dilution Assay of Long-Term Repopulating Stem Cells in the Mouse. Blood 74: 2755

26. Weilbacher, K., I. Weissman, K. Blume, and S. Heimfeld. 1991. Culture of Phenotypically Defined Hematopoietic Stem Cells and Other Progenitors at Limiting Dilution on Dexter Monolayers. Blood 78:945

27. Reincke, U., M. Rosenblatt, and S. Hellman. 1984. An *in vitro* Clonal Assay of Adherent Stem Cells (ASC) in Mouse Marrow. J. Cell. Physiol. 121:275

28. Chan, J., and F. W. Alt. 1993. Gene Rearrangement and B-Cell Development. Curr. Opin. Immunol. 5:194

29. Kincade, P. W., G. Lee, T. Watanabe, L. Sun, and M. P. Scheid. 1981. Antigens Displayed on Murine B Lymphocyte Precursors. J. Immunol. 127:2262

30. Park, Y., and D. G. Osmond. 1987. Phenotype and Proliferation of Early B Lymphocyte Precursor Cells in Mouse Bone Marrow. J. Exp. Med. 165:444

31. Osmond, D. G. 1990. B Cell Development in the Bone Marrow. Seminars in Immunol. 2:173

32. Landreth, K. S., C. Rosse, and J. Clagett. 1981. Myelogenous Production and Maturation of B Lymphocytes in the Mouse. J. Immunol. 127:2027

33. Hardy, R. R., C. E. Carmack, S. A. Shinton, J. D. Kemp, and K. Hayakawa. 1991. Resolution and Characterization of Pro-B and Pre-Pro-B Cell Stages in Normal Mouse Bone Marrow. J. Exp. Med. 173:1213

34. Lee, G., A. E. Namen, S. Gillis, L. R. Ellingsworth, and P. W. Kincade. 1989. Normal B Cell Precursors Responsive to Recombinant Murine IL-7 and Inhibition of IL-7

Activity by Transforming Growth Factor-β. J. Immunol. 142:3875

35. Suda, T., S. Okada, J. Suda, Y. Miura, M. Ito, T. Sudo, S. I. Hayashi, S. I. Nishikawa, and H. Nakauchi. 1989. A Stimulatory Effect of Recombinant Interleukin-7 on B Cell Colony Formation and an Inhibitory Effect of IL-1α. Blood 74:1936

36. Melchers, F., A. Strasser, S. R. Bauer, A. Kudo, P. Thalmann, and A. Rolink. 1989. Cellular Stages and Molecular Steps of Murine B-Cell Development. Cold Spring Harbor Symp. Quant. Biol. 54:183

37. Rolink, A., M. Streb, S. I. Nishikawa, and F. Melchers. 1991. The c-kit-Encoded Tyrosine Kinase Regulates the Proliferation of Early pre-B Cells. Eur. J. Immunol. 21:2069

38. McNiece, I. K., K. E. Langley, and K. M. Zsebo. 1991. The Role of Recombinant Stem Cell Factor in Early B Cell Development: Synergistic Interaction with IL-7. J. Immunol. 146:3785.

39. Billips, L. G., D. Petitte, K. Dorshkind, R. Narayanan, C. Chiu, and K. S. Landreth. 1992. Differential Roles of Stromal Cells, Interleukin-7, and Kit-Ligand in the Regulation of B Lymphopoiesis. Blood 79:1185

40. Landreth, K. S., R. Narayanan, and K. Dorshkind. 1992. Insulin-like Growth Factor-1 Regulates Pro-B Cell Differentiation. Blood 80:1207

41. McCulloch, E. A. 1978. Stem Cell Functions and the Clonal Haemopathies of Man. in: Stem Cells and Tissue Homeostasis. Lord, B. I., C. S. Potten, R. J. Cole (Eds,) Brit. Soc. Cell Biol. Symp. vol. 2 Cambridge Univ. Press

42. Lord, B. I., N. G. Testa, and J. H. Hendry. 1975. The Relative Spatial Distributions

of CFU-S and CFU-C in the Normal Mouse Femur. Blood 46:65

43. Lord, B. I., and J. H. Hendry. 1972. The Distribution of Haemopoietic Colony-Forming Units in the Mouse Femur and Its Modification by X-Rays. Br. J. Radiol. 45:110

44. Jacobsen, K. J. Tepper, and D. G. Osmond. 1990. Early B-Lymphocyte Precursor Cells in Mouse Bone Marrow: Subosteal Localization of B220+ Cells during Postirradiation Regeneration. Exp. Hematol. 18:304

45. Hermans, M. H. A., H. Hartsuiker, and D. Opstelten. 1989. An *in situ* Study of B-Lymphocytopoiesis in Rat Bone Marrow: Topographical Arrangement of Terminal Deoxynucleotidyl Transferase-Positive Cells and Pre-B Cells. J. Immunol. 142:67

46. Trentin, J.J. 1971. Determination of Bone Marrow Stem Cell Differentiation by Stromal Hemopoietic Inductive Microenvironments. Am. J. Pathol. 65:621

47. Silvers, W. K. 1979. The Coat Colors of Mice. Springer-Verlag.

48. Russell, E. S. 1979. Hereditary Anemias of the Mouse: A Review for Geneticists. Adv. Gen. 20:357

49. Bernstein, S. E. 1970. Tissue Transplantation as an Analytic and Therapeutic Tool in Hereditary Anemias. Am. J. Surg. 119:448

50. Dexter, T. M., and M. A. S. Moore. 1977. *In vitro* Duplication and Cure of Haemopoietic Defects in Genetically Anaemic Mice. Nature 269:412

51. Anklesaria, P., T. J. Fitzgerald, K. Kase, A. Ohara, and J. S. Greenberger. 1989. Improved Hematopoiesis in Anemic *Sl/Sl^d* Mice by Splenectomy and Therapeutic Transplantation of a Hematopoietic Microenvironment. Blood 74:1144

52. Martin, F. H., S. V. Suggs, K. E. Langley, H. S. Lu, J. Ting, K. H. Okino, C. F. Morris, I. K. McNiece, F. W. Jacobsen, E. A. Mendiaz, N. C. Birkett, M. J. Johnson, V. P. Parker, J. C. Flores, A. C. Patel, E. F. Fisher, H. O. Erjavec, C. J. Herrera, J. Wypych, R. J. Sachdev, J. A. Pope, I. Leslie, D. Wen, C. H. Lin, R. L. Cupples, and K. M. Zsebo. 1990. Primary Structure and Functional Expression of Rat and Human Stem Cell Factor DNAs. Cell 63:203

53. Copeland, N. G., D. J. Gilbert, B. C. Cho, P. J. Donovan, N. A. Jenkins, D. Cosman, D. Anderson, S. D. Lyman, and D. E. Williams. 1990. Mast Cell Growth Factor Maps near the Steel Locus on Mouse Chromosome 10 and Is Deleted in a Number of Steel Allelles. Cell 63:175

54. D. M. Anderson, S. D. Lyman, A. Baird, J. M. Wignall, J. Eisenman, C. Rauch, C. J. March, H. S. Boswell, S. D. Gimpel, D. Cosman, and D. E. Williams. 1990. Molecular Cloning of Mast Cell Growth Factor, a Hematopoietin that is Active in Both Membrane-Bound and Soluble Forms. Cell 63:235

55. Flanagan, J. G., and P. Leder. 1990. The kit Ligand: A Cell Surface Molecule Altered in Steel Mutant Fibroblasts. Cell 63:185

56. Huang, E., K. Nocka, D. R. Beier, T. Chu, J. Buck, H. Lahm, D. Wellner, P. Leder, and P. Besmer. 1990. The Hematopoietic Growth Factor KL is Encoded by the *Sl* Locus and is the Ligand of the c-kit Receptor, the Gene Product of the *W* Locus. Cell 63:225

57. Williams, D. E., J. Eisenman, A. Baird, C. Rauch, K. van Ness, C. J. March, L. S. Park, U. Martin, D. Y. Mochizuki, H. S. Boswell, G. S. Burgess, D. Cosman, and

S. D. Lyman. 1990. Identification of the Ligand for the c-kit Proto-Oncogene. Cell 63:167

58. Zsebo, K. M., J. Wypych, I. K. McNiece, H. S. Lu, K. A. Smith, S. B. Karkare, R. K. Sachdev, V. N. Yuschenkoff, N. C. Birkett, L. R. Williams, V. N. Satyagal, W. Tung, R. A. Bosselman, E. A. Mendiaz, and K. E. Langley. 1990. Identification, Purification, and Biological Characterization of Hematopoietic Stem Cell Factor from Buffalo Rat Liver-Conditioned Medium. Cell 63:195

59. Huang, E. J., K. H. Nocka, J. Buck, and P. Besmer. 1992. Differential Expression and Processing of Two Cell Associated Forms of the Kit-Ligand; KL-1 and KL-2. Mol. Biol. Cell 3:349

60. Flanagan, J. G., D. C. Chan, and P. Leder. 1991. Transmembrane Form of the Kit Ligand Growth Factor is Determined by Alternative Splicing and is Missing in the *Sl*^{d} Mutant. Cell 64:1025

61. Abboud, C. N., J. L. Liesveld, and M. A. Lichtman. 1993. The Architecture of Marrow and Its Role in Hematopoietic Celll Lodgment. in: The Hematopoietic Microenvironment: The Functional and Structural Basis of Blood Cell Development, M. W. Long and M. S. Wicha, eds. Johns Hopkins University Press.

62. Weiss, L., and H. Sakai. 1984. The Hematopoietic Stroma. Am. J. Anat. 170:447

63. Kittler, E. L. W., and P. J. Quesenberry. 1993. Stromal Cells and Clinical Implications. p49-75, in: The Hematopoietic Microenvironment: The Functional and Structural Basis of Blood Cell Development, M. W. Long and M. S. Wicha, eds. Johns Hopkins University Press.

64. Dorshkind, K. 1990. Regulation of Hemopoiesis by Bone Marrow Stromal Cells and Their Products. Ann. Rev. Immunol. 8:111

65. Weiss, L. 1976. The Hematopoietic Microenvironment of the Bone Marrow: An Ultrastructural Study of the Stroma of Rats. Anat. Rec. 186:161

66. Shaklai, M. 1989. Cellular Components of Stroma *in vivo* in Comparison with *in vitro* Systems. in: Handbook of the Hemopoietic Microenvironment M. Tavassoli (ed.) Humana Press

67. Westen, H., and D. F. Bainton. 1979. Association of Alkaline-Phosphatase-Positive Reticulum Cells in Bone Marrow with Granulocytic Precursors. J. Exp. Med. 150:919

68. Jacobsen, K., and D. G. Osmond. 1990. Microenvironmental Organization and Stromal Cell Associations of B Lymphocyte Precursor Cells in Mouse Bone Marrow. Eur. J. Immunol. 20:2395

69. King, A. G., D. Wierda, and K. S. Landreth. 1988. Bone Marrow Stromal Regulation of Lymphopoiesis: I. The Role of Macrophages, IL-1, and IL-4 in Pre-B Cell Maturation. J. Immunol. 141:2016

70. Gisler, R. H., A. Soderberg, and M. Kamber. 1987. Functional Maturation of Murine B Lymphocyte Precursors: II. Analysis of Cells Required from the Bone Marrow Microenvironment. J. Immunol. 138:2433

71. Nathan, C. F. 1987, Secretory Products of Macrophages. J. Clin. Invest. 79:319

72. Vogt, C., S. Pentz, and I. Rich. 1989. A Role for the Macrophage in Normal Hemopoiesis: III. *in vitro* and *in vivo* Erythropoietin Gene Expression in Macrophages Detected by *in situ* Hybridization. Exp. Hematol. 17:391

73. Deldar, A., H. Lewis, and L. Weiss. 1985. Bone Lining Cells and Hemopoiesis: An Electron Microscopic Study of Canine Bone Marrow. Anat. Rec. 213:187

74. Weiss, L., and U. Geduldig. 1991. Barrier Cells: Stromal Regulation of Hematopoiesis and Blood Cell Release in Normal And Stressed Murine Bone Marrow. Blood 78:975

75. Chan, S. H., and D. Metcalf. 1972. Local Production of Colony-Stimulating Factor within the Bone Marrow: Role of Non-Hematopoietic Cells. Blood 40:646

76. Horton, M. A., E. F. Rimmer, D. Lewis, J. A. S. Pringle, K. Fuller, and T. J. Chambers. 1984. Cell Surface Characterization of the Human Osteoclast: Phenotypic Relationship to Other Bone Marrow-Derived Cell Types. J. Pathol. 144:281

77. Kodama, H., A. Yamasaki, M. Nose, S. Niida, Y. Ohgame, M. Abe, M. Kumegawa, and T. Suda. 1991. Congenital Osteoclast Deficiency in Osteopetrotic (*op/op*) Mice is Cured by Injections of Macrophage Colony-Stimulating Factor. J. Exp. Med. 173:269

78. Shinar, D., and G. A. Rodan. 1993. Relationships and Interactions between Bone and Bone Marrow. in: The Hematopoietic Microenvironment: The Functional and Structural Basis of Blood Cell Development, M. W. Long and M. S. Wicha, eds. Johns Hopkins University Press.

79. Chambers, T. J. 1980. The Cellular Basis of Bone Resorption. Clin. Orthop. Rel. Res. 152:283

80. Nijweidi, P. J., A. van der Plas, and A. A. Olthof. 1988. Osteoblastic Differentiation. Cell and Molecular Biology of Vertebrate Hard Tissues. Wiley,

Chicester

81. Weinreb, M., D. Shinar, and G. A. Rodan. 1990. Different Pattern of Alkaline Phosphatase, Osteopontin, and Osteocalcin Expression in Developing Rat Bone Visualized by *in situ* Hybridization. J. Bone Miner. Res. 5:831

82. Rodan, G. A., J. K. Heath, K. Yoon, M. Noda, and S. B. Rodan. 1988. Diversity of the Osteoblastic Phenotype. Cell and Molecular Biology of Vertebrate Hard Tissues. Wiley, Chicester

83. Sahebekhtiari, H. A., and M. Tavassoli. 1978. Studies on Bone Marrow Hitogenesis: Morphometric and Autoradiographic Studies of Regenerating Marrow Stroma in Extramedullary Autoimplants and after Evacuation of Marrow Cavity. Cell Tiss. Res. 192:437

84. Fan, H. R. Yasumizu, K. Sugiura, N. Oyaizu, Y. Ohnishi, F. Takao, M. Inaba, J. Liu, and S. Ikehara. 1990. Histogenesis of Hemopoietic Bone Marrow in Adult Mice. Exp. Hematol. 18:159

85. Tavassoli, M., and W. H. Crosby. 1968. Transplantation of Marrow to Extramedullary Sites. Science 161:54

86. Maniatis, A., M. Tavassoli, and W. H. Crosby. 1971. Origin of Osteogenic Precursor Cells in Extramedullary Marrow Implants. Blood 38:569

87. Bleiberg, I., M. D. Ricciardone, H. A. Reddi, and K. F. McCarthy. 1987. New Bone Formation and Bone Marrow Differentiation Induced in Rats by Extracellular Bone Matrix Implantation: Effect of Local Preirradiation on the Process. Exp.Hematol. 15:309

88. Giuliani, D. C., J. C. Hall, and B. S. Morse. 1986. Strategies of Hemopoietic Stress Adaptation within the Medullary Cavity. Anat. Rec. 216:528

89. Alavi, A., J. P. Bond, D. E. Kuhl, and R. H. Creech. 1974. Scan Detection of Bone Marrow Infarcts in Sickle Cell Disorders. J. Nucl. Med. 15:1003

90. Bohrer, S. P. 1970. Acute Long Bone Diaphyseal Infarcts in Sickle Cell Disease. Br. J. Radiol. 43:685

91. Choremis, C., D. Liakakos, C. Tseghi, and C. Moschovakis. 1965. Pathogenesis of the Osseous Lesions in Thalassemia. J. Pediatr. 66:962

92. Gratwick, G. M., P. G. Bullough, W. H. O. Bohne, A. L. Markenson, and C. M. Peterson. 1978. Thalassemic Osteoarthropathy. Ann. Intern. Med. 88:494

93. Jee, W. S. S., T. J. Wronski, E. R. Morey, and D. B. Kimmel. 1983. Effects of Spaceflight on Trabecular Bone in Rats. Am J. Physiol. 244:R310

94. Tavassoli, M. 1982. Anemia of Spaceflight. Blood 60:1059

95. Minaire, P. C. Eduard, M. Arlot, and P. J. Meunier. 1984. Marrow Changes in Paraplegic Patients. Calcif. Tiss. Intl. 36:338

96. Perkins, S., and R. A. Fleischman. 1988. Hematopoietic Microenvironment: Origin, Lineage, and Transplantability of the Stromal Cells in Long-term Bone Marrow Cultures from Chimeric Mice. J. Clin. Invest. 81:1072

97. Lennon, J. E., and H. S. Micklem. 1986. Stromal Cells in Long-Term Murine Bone Marrow Culture: FACS Studies and Origin of Stromal Cells in Radiation Chimeras. Exp. Hematol. 14:287

98. Simmons, P. J., D. Przepiorka, E. D. Thomas, and B. Torok-Storb. 1987. Host

Origin of Marrow Stromal Cells Following Allogeneic Bone Marrow Transplantation. Nature 328:429-432

99. Huang, S., and L. W. M. M. Terstappen. 1992. Formation of Haematopoietic Microenvironment and Haematopoietic Stem Cells from Single Human Bone Marrow Stem Cells. Nature 360:745

100. Dexter, M., and T. Allen. 1992. Multi-Talented Stem Cells? Nature 360:709

101. Simmons, P. J., and B. Torok-Storb. 1991. CD34 Expression by Stromal Precursors in Normal Human Adult Bone Marrow. Blood 78:2848

102. Grigoriadis, A. E., J. N. M. Heersche, and J. E. Aubin. 1988. Differentiation of Muscle, Fat, Cartilage, and Bone from Progenitor Cells Present in a Bone-Derived Clonal Cell Population: Effect of Dexamethasone. J. Cell Biol. 106:2139

103. Friedenstein, A. J., R. K. Chailakhjan, and K. S. Lalykina. 1970. The Development of Fibroblast Colonies in Monolayer Cultures of Guinea-Pig Bone Marrow and Spleen Cells. Cell Tiss. Kinet. 3:393

104. Dexter, T. .M, T. D. Allen, and L. G. Lajtha. 1977. Conditions Controlling the Proliferation of Haemopoietic Stem Cells *in vitro*. J. Cell Physiol. 91:335

105. Whitlock, C. A., D. Robertson, and O. N. Witte. 1984. Murine B Cell Lymphopoiesis in Long-Term Culture. J. Immunol. Methods 67:353

106. Whitlock, C. A., and O. N. Witte. 1982. Long-Term Culture of B Lymphocytes and Their Precursors from Murine Bone Marrow. Proc. Natl. Acad. Sci. USA 79:3608

107. Hocking, W. G., and D. W. Golde. 1980. Long-Term Human Bone Marrow Cultures. Blood 56:118

108. Wolf, M. L., J. A. Buckley, A. Goldfarb, C. Law, and T. W. LeBien. 1991. Development of a Bone Marrow Culture for Maintenance and Growth of Normal Human B Cell Precursors. J. Immunol. 147:3324

109. Hasthorpe, S., S. L. Green, J. Rogerson, and J. M. Radley. 1991. A Mouse Endothelial Cell-Specific Monoclonal Antibody: Its Reactivity with LTMC Endothelium. Exp. Hematol. 19:166

110. Witte, P. L., M. Robinson, A. Henley, M. G. Low, D. L. Stiers, S. Perkins, R. A. Fleischman, and P. W. Kincade. 1987. Relationships Between B-Lineage Lymphocytes and Stromal Cells in Long-Term Bone Marrow Cultures. Eur. J. Immunol. 17:1473

111. Dorshkind, K., L. Schouest, and W. H. Fletcher. 1985. Morphologic Analysis of Long-Term Bone Marrow Cultures that Support B-Lymphopoiesis or Myelopoiesis. Cell Tiss. Res. 239:375

112. Kierney, P. C., and K. Dorshkind. 1987. B Lymphocyte Precursors and Myeloid Progenitors Survive in Diffusion Chamber Cultures but B Cell Differentiation Requires Close Association with Stromal Cells. Blood 70:1418

113. Miyake, K., K. Medina, K. Ishihara, M. Kimoto, R. Auerbach, and P. W. Kincade. 1991. A VCAM-like Adhesion Molecule on Bone Marrow Stromal Cells Mediates Binding of Lymphocyte Precursors in Culture. J. Cell Biol. 114:557

114. Miyake, K., K. L. Medina, S. I. Hayashi, S. Ono, T. Hamaoka, and P. W. Kincade. 1990. Monoclonal Antibodies to pgp-1/CD44 Block Lympho-Hemopoiesis in Long-Term Bone Marrow Cultures. J. Exp. Med. 171:477

115. Imhof, B. A., C. Schlienger, K. Handloser, B. Hesse, M. Slanicka, and R. Gisler. 1991. Monoclonal Antibodies that Block Adhesion of B Cell Progenitors to Bone Marrow Stroma *in vitro* Prevent B Cell Differentiation *in vivo*. Eur. J. Immunol. 21:2043

116. Miyake, K., I. L. Weissman, J. S. Greenberger, and P. W. Kincade. 1991. Evidence for a Role of the Integrin VLA-4 in Lympho-Hemopoiesis. J. Exp. Med. 173:599

117. Miyake, K., C. B. Underhill, J. Lesley, and P. W. Kincade. 1990. Hyaluronate can Function as a Cell Adhesion Molecule and CD44 Participates in Hyaluronate Recognition. J. Exp. Med. 172:69

118. Simmons, P. J., B. Masinovsky, B. M. Longenecker, R. Berenson, B. Torok-Storb, and W. M. Gallatin. 1992. Vascular Cell Adhesion Molecule-1 Expressed by Bone Marrow Stromal Cells Mediates the Binding of Hematopoietic Progenitor Cells. Blood 80:388

119. Ryan, D. H., B. L. Nuccie, C. N. Abboud, and J. M. Winslow. 1991. Vascular Cell Adhesion Molecule-1 and the Integrin VLA-4 Mediate Adhesion of Human B Cell Precursors to Cultured Bone Marrow Adherent Cells. J. Clin. Invest. 88:995

120. Watson, J. D., and H. J. McKenna. 1992. Novel Factors from Stromal Cells: Bone Marrow and Thymus Microenvironments. Intl. J. Cell Clon. 10:144

121. Witte, P. L., L. M. Frantsve, M. Hergott, and S. M. Rahbe. 1993. Cytokine Production and Heterogeneity of Primary Stromal Cells that Support B-Lymphopoiesis. Eur. J. Immunol. 23:1809-1817

122. Singer, J. W., J. L. Slack, M. B. Lilly, and D. F. Andrews. 1993. Marrow

Stromal Cells: Response to Cytokines and Control of Gene Expression. in: The Hematopoietic Microenvironment: The Functional and Structural Basis of Blood Cell Development, M. W. Long and M. S. Wicha, eds. Johns Hopkins University Press.

123. Dexter, T. M. L. H. Coutinho, E. Spooncer, C. M. Heyworth, C. P. Daniel, R. Schiro, J. Chang, and T. D. Allen. 1989. Stromal Cells in Haemopoiesis. Ciba Found. Symp. 148:76

124. Gualtieri, R. J., C. Liang, R. K. Shadduck, A. Waheed, and J. Banks. 1987. Identification of the Hematopoietic Growth Factors Elaborated by Bone Marrow Stromal Cells Using Antibody Neutralization Analysis. Exp. Hematol. 15:883-889

125. Williams, D. A., M. F. Rosenblatt, D. R. Beier, and R. D. Cone. 1988. Generation of Murine Stromal Cell Lines Supporting Hematopoietic Stem Cell Proliferation by Use of Recombinant Retrovirus Vectors Encoding Simian Virus 40 Large T Antigen. Mol. Cell. Biol. 8:3864-3871

126. Rios, M., and D. A. Williams. 1990. Systematic Analysis of the Ability of Stromal Cell Lines from Different Murine Adult Tissues to Support Maintenance of Hematopoietic Stem Cells *in vitro*. J. Cell. Physiol. 145:434-443

127. Henderson, A. J., A. Johnson, and Kenneth Dorshkind. 1990. Functional Characterization of Two Stromal Cell Lines that Support B Lymphopoiesis. J. Immunol. 145:423

128. Nishikawa, S. I., M. Ogawa, S. Nishikawa, T. Kunisada, and H. Kodama. 1988. B Lymphopoiesis on Stromal Cell Clone: Stromal Cell Clones Acting on Different Stages of B Cell Differentiation. Eur. J. Immunol. 18:1767

129. Zipori, D., D. Duskin, M. Tamir, A. Argaman, J. Toledo, and Z. Malik. 1985. Cultured Mouse Marrow Stromal Cell Lines. II. Distinct Subtypes Differing in Morphology, Collagen Types, Myelopoietic Factors, And Leukemic Cell Growth Modulating Activities. J. Cell. Physiol. 122:81

130. Lanotte, M. D. Scott, T.M. Dexter, and T. D. Allen. 1982. Clonal Preadipocyte Cell Lines with Different Phenotypes Derived from Murine Marrow Stroma: Factors Influencing Growth and Adipogenesis *in vitro*. J. Cell. Physiol. 111:177

131. Hunt, P., D. Robertson, D. Weiss, D. Rennick, F. Lee, and O. N. Witte. 1987. A Single Bone Marrow-Derived Stromal Cell Type Supports the *in vitro* Growth of Early Lymphoid and Myeloid Cells. Cell 48:997

132. Pietrangeli, C. E., S. I. Hayashi, and P. W. Kincade. 1988. Stromal Cell Lines Which Support Lymphocyte Growth: Characterization, Sensitivity to Radiation and Responsiveness to Growth Factors. Eur. J. Immunol. 18:863

133. Whitlock, C. A., G. F. Tidmarsh, C. Muller-Sieburg, and I. L. Weissman. 1987. Bone Marrow Stromal Cell Lines with Lymphopoietic Activity Express High Levels of a Pre-B Neoplasia-Associated Molecule. Cell 48:1009-1021

134. Gimble, J. M., M. A. Dorheim, Q. Cheng, K. Medina, C. S. Wang, R. Jones, E. Koren, C. Pietrangeli, and P. W. Kincade. 1990. Adipogenesis in a Murine Bone Marrow Stromal Cell Line Capable of Supporting B Lineage Lymphocyte Growth and Proliferation: Biochemical and Molecular Characterization. Eur. J. Immunol. 20:379

135. Collins, L. S., and K. Dorshkind. 1987. A Stromal Cell Line from Myeloid Long-Term Bone Marrow Cultures can Support Myelopoiesis and B Lymphopoiesis. J.

Immunol. 138:1082

136. Lee, K. H., T. Kinashi, K. Tohyama, K. Tashiro, N. Funato, K. Hama, and T. Honjo. 1991. Different Stromal Cell Lines Support Lineage-Selective Differentiation of the Multipotential Bone Marrow Stem Cell Clone LyD9. J. Exp. Med. 173:1257

137. Zipori, D. 1988. Hemopoietic Microenvironments. in: Hematopoiesis: Long-Term Effects on Chemotherapy and Radiation. N. G. Testa and R. P. Gale (Eds.) Marcel Dekker publishers 1988

138. Charbord, P., H. Lerat, I. Newton, E. Tamayo, A. M. Gown, J. W. Singer, and P. Herve. 1990. The Cytoskeleton of Stromal Cells from Human Bone Marrow Cultures Resembles that of Cultured Smooth Muscle Cells. Exp. Hematol. 18:276

139. Charbord, P., A. M. Gown, A. Keating, and J. W. Singer. 1985. CGA-7 and HHF, Two Monoclonal Antibodies that Recognize Muscle Actin and React with Adherent Cells in Human Long-Term Bone Marrow Cultures. Blood 66:1138

140. Peled, A., D. Zipori, O. Abramsky, H. Ovadia, and Elias Shezen. 1991. Expression of α-Smooth Muscle Actin in Murine Bone Marrow Stromal Cells. Blood 78:304

141. Johnson, A., and K. Dorshkind. 1986. Stromal Cells in Myeloid and Lymphoid Long-Term Bone Marrow Cultures Can Support Multiple Hemopoietic Lineages and Modulate Their Production of Hemopoietic Growth Factors. Blood 68:1348

142. Witte, P. L., P. W. Kincade, and V. Vetvicka. 1986. Interculture Variation and Evolution of B Lineage Lymphocytes in Long-Term Bone Marrow Culture. Eur. J. Immunol. 16:779

143. Landreth, K. S., and K. Dorshkind. 1988. Pre-B Cell Generation Potentiated by Soluble Factors from a Bone Marrow Stromal Cell Line. J. Immunol. 140:845

144. Crocker, P. R., and S. Gordon. 1985. Isolation and Characterization of Resident Stromal Macrophages and Hematopoietic Cell Clusters from Mouse Bone Marrow. J. Exp. Med. 162:993

145. Blaszek, I., J. Misset, M. Benavides, M. Comisso, P. Ribaud, and G. Mathe. 1990. Hematon, a Multicellular Functional Unit in Normal Human Bone Marrow: Structural Organization, Hemopoietic Activity, and Its Relationship to Myelodysplasia and Myeloid Leukemias. Exp. Hematol. 18:259

146. Kina, T., A. S. Majumdar, S. Heimfeld, H. Kaneshima, B. Holzmann, Y. Katsura, and I. L. Weissman. 1991. Identification of a 107-Kd Glycoprotein that Mediates Adhesion between Stromal Cells and Hematolymphoid Cells. J. Exp. Med. 173:373

147. Jacobsen, K., K. Miyake, P. W. Kincade, and D. G. Osmond. 1992. Highly Restricted Expression of a Stromal Cell Determinant in Mouse Bone Marrow *in vivo*. J. Exp. Med. 176:927

148. Osborn, L., C. Hession, R. Tizard, C. Vassallo, S. Luhowskyj, G. Chi-Rosso, and R. Lobb. 1989. Direct Expression Cloning of Vascular Cell Adhesion Molecule 1, a Cytokine-Induced Endothelial Protein that Binds to Lymphocytes. Cell 59:1203

149. Elices, M. J., L. Osborn, Y. Takada, C. Crouse, S. Luhowskyj, M. E. Hemler, and R. R. Lobb. 1990. VCAM-1 on Acitivated Endothelium Interacts with the Leukocyte Integrin VLA-4 at a Site Distinct from the VLA-4/Fibronectin Binding Site. Cell 60:577

150. Bevilacqua, M. P. 1993. Endothelial-Luekocyte Adhesion Molecules. Ann. Rev.

Immunol. 11:767

151. Cumano, A., K. Dorshkind, S. Gillis, and C. J. Paige. 1990. The Influence of S17 Stromal Cells and Interleukin-7 on B Cell Development. Eur. J. Immunol. 20:2183

152. Kadouri, A., R. Kompier, J. Honigwachs-Shaanani, J. Toledo, N. Brosh, D. Sternberg, A. Levy, E. Tzehoval, and D. Zipori. 1992. Dynamic Changes in Cytokine Secretion by Stromal Cells During Prolonged Maintenance under Protein-Free Conditions. Int. J. Cell Clon. 10:299

153. McGrath, H. E., C. Liang, T. A. Alberico, and P. J. Quesenberry. 1987. The Effect of Lithium on Growth Factor Production in Long-Term Bone Marrow Cultures. Blood 70:1136

154. Witte, P. L., L. M. Frantsve, X. Giao, and G. Perry. 1993. Similarities in Cytokine Gene Transcription by Stromal Cells under Lymphopoietic or Myelopoietic Culture Conditions. J. Immunol. 150:21A (abstract)

155. Kodama, H., H. Hagiwara, H. Sudo, Y. Amagai, T. Yokota, N. Arai, and Y. Kitamura. 1986. MC3T3/PA6 Preadipocytes Support *in vitro* Proliferation of Hemopoietic Stem Cells Through a Mechanism Different from that of Interleukin-3. J.Cell. Physiol. 129:20

156. Ogilvie, A. D., N. C. Wood, E. Dickens, D. Wojtacha, and G. W. Duff. 1990. *in situ* Hybridization. Ann. Rheum. Dis. 49:434

157. Dallman, M. J., R. A. Montgomery, C. P. Larsen, A. Wanders, and A. F. Wells. 1991. Cytokine Gene Expression: Analysis using Northern Blotting, Polymerase Chain Reaction, and *in situ* Hybridization. Immunol. Revs. 119:163

158. Shirai, A., K. Holmes, and D. Klinman. 1993. Detection and Quantitation of Cells Secreting IL-6 under Physiologic Conditions in BALB/c Mice. J. Immunol. 150:793

159. Namen, A. E., S. Lupton, K. Hjerrild, J. Wignall, D. Y. Mochizuki, A. Schmierer, B. Mosley, C. J. March, D. Urdal, S. Gillis, D. Cosman, and R. G. Goodwin. 1988. Stimulation of B-Cell Progenitors by Cloned Murine Interleukin-7. Nature 333:571

160. Morrissey, P. J., P. Conlon, S. Braddy, D. E. Williams, A. E. Namen, and D. Y. Mochizuki. 1991. Adminstration of IL-7 to Mice with Cyclophosphamide-Induced Lymphopenia Accelerates Lymphocyte Repopulation. J. Immunol. 146:1547

161. Grabstein, K. H., T. J. Waldschmidt, F. D. Finkelman, B. W. Hess, A. R. Alpert, N. E. Boiani, A. E. Namen, and P. J. Morrissey. 1993. Inhibition of Murine B and T Lymphopoiesis *in vivo* by an Anti-Interleukin-7 Monoclonal Antibody. J. Exp. Med. 178:257

162. Ogawa, M., Y. Matsuzaki, A. Nishikawa, S. Hayashi, T. Kunisada, T. Sudo, T. Kina, H. Nakauchi, and S. Nishikawa. 1991. Expression and Function of c-kit in Hemopoietic Progenitor Cells. J. Exp. Med. 174:63

163. Wiktor-Jedrzejczak, W., A. Bartocci, A. W. Ferrante, A. Ahmed-Ansari, K. W. Sell, J. W. Pollard, and E. R. Stanley. 1990. Total Absence of Colony-Stimulating Factor-1 in the Macrophage-Deficient Osteopetrotic (*op/op*) Mouse. Proc. Natl. Acad. Sci. USA 87:4828

164. Wiktor-Jedrzejczak, W., A. Ahmed, C. Szczylik, and R. R. Skelly. 1982. Hematological Characterization of Congenital Osteopetrosis in *op/op* Mouse. J. Exp.

Med. 156:1516

165. Tavassoli, M. and J. M. Yoffey. 1983. Bone Marrow Structure and Function. A. R. Liss, New York

166. Gimble, J. M., C. Pietrangeli, A. Henley, M. A. Dorheim, J. Silver, A. Namen, M. Takeichi, C. Goridis, and P. W. Kincade. 1989. Characterization of Murine Bone Marrow and Spleen-Derived Stromal Cells: Analysis of Leukocyte Marker and Growth Factor mRNA Transcript Levels. Blood 74:303

167. Witte, P. L., P. W. Kincade, and V. Vetvicka. 1986. Interculture Variation and Evolution of B Lineage Lymphocytes in Long-Term Bone Marrow Culture. Eur. J. Immunol. 16:779

168. Wekerle, H., and U. Ketelsen. 1980. Thymic Nurse Cells- Ia-Bearing epithelium Involved in T-Lymphocyte Differentiation? Nature 283:402

169. Fei, R, P. E. Penn, and N. S. Wolf. 1990. A Method to Establish Pure Fibroblast and Endothelial Cell Colony Cultures from Murine Bone Marrow. Exp. Hematol. 18:953

170. Striker, G. E., J. M. Harlan, and S. M. Schwartz. 1980. Human Endothelial Cells *in vitro*. Meth. Cell Biol. 21A:135-151

171. Miller, B. A., G. Antognetti, and T. A. Springer. 1985. Identification of Cell Surface Antigens Present on Murine Hematopoietic Stem Cells. J. Immunol. 134:3286

172. Cooper, M. D., D. Mulvaney, A. Coutinho, and P. Cazenave. 1986. A Novel Cell Surface Molecule on Early B Lineage Cells. Nature 321:616

173. Park, Y., and D. G. Osmond. 1989. Dynamics of Early B Lymphocyte Precursor Cells in Mouse Bone Marrow: Proliferation of Cells Containing Terminal

Deoxynucleotidyl Transferase. Eur. J. Immunol. 19:2139

174. Opstelten, D., and D. G. Osmond. 1983. Pre-B Cells in Mouse Bone Marrow: Immunofluorescence Stathmokinetic Studies of the Proliferation of Cytoplasmic μ-Chain-Bearing Cells in Normal Mice. J. Immunol. 131:2635

175. Opstelten, D., and D. G. Osmond. 1985. Regulation of Pre-B Cell Proliferation in Bone Marrow: Immunofluorescence Stathmokinetic Studies of Cytoplasmic μ Chain-Bearing Cells in Anti-IgM-Treated Mice, Hematologically Deficient Mutant Mice and Mice Given Sheep Red Blood Cells. Eur. J. Immunol. 15:599

176. Landreth, K. S., P. W. Kincade, G. Lee, and D. E. Harrison. 1984. B Lymphocyte Precursors in Embryonic and Adult *W* Anemic Mice. J. Immunol. 132:2724

177. Muller-Sieburg, C. E., C. A. Whitlock, and I. L. Weissman. 1986. Isolation of Two Early B Lymphocyte Progenitors from Mouse Marrow: a Committed Pre-Pre-B Cell and a Clonogenic Thy-1^{lo} Hematopoietic Stem Cell. Cell 44:653

178. Lefkovits, I., and H. Waldmann. 1984. Limiting Dilution Analysis of the Cells of the Immune System I. The Clonal Basis of the Immune Response. Immunol. Today 5:265

179. Fazekas de St. Groth, S. 1982. The Evaluation of Limiting Dilution Assays. J. Immunol. Meth. 49:R11

180. de Vries, P., K. A. Brasel, J. R. Eisenman, A. R. Alpert, and D. E. Williams. 1991. The Effect of Recombinant Mast Cell Growth Factor on Purified Murine Hematopoietic Stem Cells. J. Exp. Med. 173:1205

181. Broxmeyer, H. E., G. Hangoc, S. Cooper, D. Anderson, D. Cosman, S. D. Lyman, and D. E. Williams. 1991. Influence of Murine Mast Cell Growth Factor (c-kit Ligand) on Colony Formation by Mouse Marrow Hematopoietic Progenitor Cells. Exp. Hematol. 19:143

182. Broxmeyer, H. E., S. Cooper, L. Lu, G. Hangoc, D. Anderson, D. Cosman, S. D. Lyman, and D. E. Williams. 1991. Effect of Murine Mast Cell Growth Factor (c-kit Proto-Oncogene Ligand) on Colony Formation by Human Marrow Hematopoietic Progenitor Cells. Blood 77:2142

183. Jackson, C. J., P. K. Garbett, B. Nissen, and L. Schrieber. 1990. Binding of Human Endothelium to *Ulex Europaeus-I* Coated Dynabeads: Application to the Isolation of Microvascular Endothelium. J. Cell Sci. 96:257

184. Ishibashi, T., S. L. Miller, and S. A. Burstein. 1987. Type β Transforming Growth Factor is a Potent Inhibitor of Murine Megakaryocytopoiesis *in vitro*. Blood 69:1737

185. Sudo, H., H. Kodama, Y. Amagai, S. Yamamoto, and S. Kasai. 1983. *In vitro* Differentiation and Calcification in a New Clonal Osteogenic Cell Line Derived from Newborn Mouse Calvaria. J. Cell Biol. 96:191

186. Quarles, L. D., D. A. Yohay, L. W. Lever, R. Caton, and R. J. Wenstrup. 1992. Distinct Proliferative and Differentiated Stages of Murine MC3T3-E1 Cells in Culture: An *in vitro* Model of Osteoblast Development. J. Bone Min. Res. 7:683

187. Yam, L. T., C. Y. Li, and W. H. Crosby. 1971. Cytochemical Identification of Monocytes and Granulocytes. Am. J. Clin. Pathol. 55:283

188. Li, C. Y., K. W. Lam, and L. T. Yam. 1973. Esterases in Human Leukocytes. J.

Histochem. Cytochem. 21:1

189. Galmiche, M. C. V. E. Koteliansky, J. Briere, P. Herve, and P. Charbord. 1993. Stromal Cells from Human Long-Term Marrow Cultures are Mesenchymal Cells that Differentiate Following a Vascular Smooth Muscle Differentiation Pathway. Blood 82:66-76

190. Herman, I. M., and P. A. D'Amore. 1985. Microvascular Pericytes Contain Muscle and Nonmuscle Actins. J. Cell Biol. 101:43

191. Sims, D. E. 1986. The Pericyte-A Review. Tissue and Cell 18:153-174

192. Lanotte, M., S. Schorr, and T. M. Dexter. 1981. Collagen Gels as a Matrix for Haemopoiesis. J. Cell. Physiol. 106:269

193. Rosen, G. D., J. R. Sanes, R. LaChance, J. M. Cunningham, J. Roman, and D. C. Dean. 1992. Roles for the Integrin VLA-4 and Its Counter Receptor VCAM1 in Myogenesis. Cell 69:1107

194. Weibel, E. R. 1974. On Pericytes, Particularly Their Existence on Lung Capillaries. Microvasc. Res. 8:218

195. Osmond, D. G., K. Miyake, P. W. Kincade, and K. Jacobsen. 1993. *In vivo* Expression of the Adhesion Molecule VCAM1 and the Integrin VLA-4 in Normal and Gamma-Irradiated Mouse Bone Marrow. J. Immunol. 150:15A (abstract)

196. Holthofer, H., I. Virtanen, A. L. Kariniemi, M. Hormia, E. Linder, and A. Miettinen. 1982. *Ulex europoaeus I* Lectin as a Marker for Vascular Endothelium in Human Tissues. Lab. Invest. 47:60-66

197. Walker, R. A. 1985. *Ulex europaeus I*-Peroxidase as a Marker of Vascular

Endothelium: Its Application in Routine Histopathology. J. Pathol. 146:123-127

198. Miettinen, M., H. Holthofer, V. Lehto, A. Miettinen, and I. Virtanen. 1983. *Ulex europaeus I* Lectin as a Marker for Tumors Derived from Endothelial Cells.

199. Pino, R. M. 1984. Ultrastructural Localization of Lectin Receptors on the Bone Marrow Sinusoidal Endothelium of the Rat. Am. J. Anat. 169:259

200. Soda, R., and M. Tavassoli. 1983. Mapping of the Bone Marrow Sinus Endothelium with Lectins and Glycosylated Ferritins: Identification of Differentiated Microdomains and Their Functional Significance. J. Ultrastruc. Res. 84:299

201. Auerbach, R., L. Alby, J. Grieves, J. Joseph, C. Lindgren, L. W. Morrissey, Y. A. Sidky, M. Tu, and S. L. Watt. 1982. Monoclonal Antibody Against Angiotensin-Converting Enzyme: Its Use as a Marker for Murine, Bovine, and Human Endothelial Cells. Proc. Natl. Acad. Sci. USA 79:7891

202. Jonsson, J., and R. A. Phillips. 1993. Interleukin-7 Responsiveness of B220+ B Cell Precursors from Bone Marrow Decreases in Aging Mice. Cell. Immunol. 147:267

203. Rolink, A., D. Haasner, S. Nishikawa, and F. Melchers. 1993. Changes in Frequencies of Clonable Pre-B Cells During Life in Different Lymphoid Organs of Mice. Blood 81:2290

204. Heinrich, M. C., D. C. Dooley, A. C. Freed, L. Band, M. E. Hoatlin, W. W. Keeble, S. T. Peters, K. V. Silvey, F. S. Ey, D. Kabat, R. T. Maziarz, and G. C. Bagby. 1993. Constitutive Expression of Steel Factor Gene by Human Stromal Cells. Blood 82:771

205. Vetvicka, V., P. W. Kincade, and P. L. Witte. 1986. Effects of 5-Fluorouracil on

B Lymphocyte Lineage Cells. J. Immunol. 137:2405

206. Williams, D. A., M. Rios, C. Stephans, and V. P. Patel. 1991. Fibronectin and VLA-4 in Hematopoietic Stem Cell-Microenvironment Interactions. Nature 352:438

207. Gallagher, R. B., and D. G. Osmond. 1991. To B, or not to B: That is the Question. Immunol. Today 12:1

208. Shackney, S. E., S. S. Ford, and A. B. Wittig. 1975. Kinetic-Microarchitectural Correlations in the Bone Marrow of the Mouse. Cell Tissue Kinet. 8:505

209. Muller-Sieburg, C. E. 1991. Separation of Pluripotent Stem Cells and Early B Lymphocyte Precursors with Antibody Fall-3. J. Exp. Med. 174:161

210. Palacios, R., and J. Samaridis. 1992. Fetal Liver Pro-B and Pre-B Lymphocyte Clones: Expression of Lymphoid-Specific Genes, Surface Markers, Growth Requirements, Colonization of the Bone Marrow, and Generation of B Lymphocytes *in vivo* and *in vitro*. Mol. Cell. Biol. 12:518

211. Mekori, T., and R. A. Phillips. 1969. The Immune Response in Mice of Genotypes *W/W^v* and *Sl/SL^d*. Proc. Soc. Exp. Biol. Med. 132:115

212. Ha, T., N. D. Reed, and P. K. Crowle. 1986. Immune Response Potential of Mast Cell-Deficient *W/W^v* Mice. Int. Archs. Allergy Appl. Immun. 80:85

213. Shearer, G. M., and G. Cudkowicz. 1967. Deficient Production of Antibody-Forming Cells by Genetically Anemic *W^{v}W^v* and *W^v*+ Mice (abstr). Fed. Proc. 26:688

214. Gordon, M. Y., G. P. Riley, S. M. Watt, and M. F. Greaves. 1987. Compartmentalization of a Haematopoietic Growth Factor (GM-CSF) by Glycosaminoglycans in the Bone Marrow Microenvironment. Nature 326:403

215. Roberts, R., J. Gallagher, E. Spooncer, T. D. Allen, F. Bloomfield, and T. M. Dexter. 1988. Heparan Sulphate Bound Growth Factors: a Mechanism for Stromal Cell Mediated Haemopoiesis. Nature 332:376

VITA

The author, Phillip E. Funk, was born in Jerseyville, Illinois on March 15, 1965 to Paul and Rosalie Funk. He received his secondary education at Jersey Community High School, graduating in 1983. His undergraduate education was at the University of Illinois in Urbana-Champaign, where he earned a Bachelor of Science degree in Microbiology in 1987.

In August of 1988 Phil entered the Department of Microbiology in the Graduate School of Loyola University. In February of 1990 he joined the laboratory of Dr. Pamela Witte where he began his studies on bone marrow stromal cells that are involved in B lymphopoiesis. Phil was the recipient of a Loyola University Dissertation Fellowship in 1992 and was awarded the outstanding student presentation at the Autumn Immunology Conference in November of 1992. Upon completing his studies in Dr. Witte's lab, Phil will begin post-doctoral work in the laboratory of Dr. Craig Thompson at the Howard Hughes Medical Institute at the University of Chicago, where he will study molecular aspects of lymphocyte differentiation. Phillip is married to Wendy E. Funk.

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The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY.

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